

APPLICATION
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TITLE: COMPOSITIONS AND METHODS FOR INHIBITING G2
CELL CYCLE ARREST AND SENSITIZING CELLS TO
DNA DAMAGING AGENTS

APPLICANT: Masashi Suganuma, a citizen of Japan
Residing at 1-1-16 Shibuya-cho, Toyota City, Japan 471-0808

Takumi Kawabe, a citizen of Japan
Residing at 1-44-1 Mikanyama-cho, Mizuho-ku, Nagoya City, Japan 467-0041

ASSIGNEE: CanBas Co., Ltd.
709-1 Aza Takanomisaki Sanmaibashi, Numazu-city, Shizuoka, Japan 410-0031

Fish & Richardson P.C.
4350 La Jolla Village Drive, Suite 500
San Diego, CA 92122
Tel.: (858) 678-5070
Fax: (858) 678-5099

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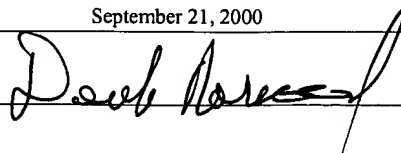
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COMPOSITIONS AND METHODS FOR INHIBITING G2 CELL CYCLE ARREST AND SENSITIZING CELLS TO DNA DAMAGING AGENTS

TECHNICAL FIELD

This invention generally pertains to the fields of medicine and cancer therapeutics. In particular, this invention provides novel genes and polypeptides and methods for making and using them. Specifically, the compositions and methods of the invention are used to treat disorders of cell growth, such as cancer. In particular, the invention provides methods for selectively sensitizing G1 checkpoint impaired cancer cells to DNA damaging agents and treatments. Also provided are methods for screening for compounds able to interact with, e.g., inhibit, enzymes involved in the G2 cell cycle arrest checkpoint, such as Chk1 and/or Chk2/Cds1 kinase.

BACKGROUND

It is a continuing challenge to develop anti-cancer agents that are capable of inhibiting the growth of, or killing, cancer cells, without affecting normal cells. Researchers have focused on genetic mutations in cancer cells to find clues to discover such new anti-cancer drugs.

Many cancer cells have mutations in genes involved in the G1 cell cycle arrest checkpoint. Such genes include impaired tumor suppressor genes, e.g., p53, Rb, p16^{INK4}, and p19^{ARF}. Alternatively, such mutations can cause expression of oncogenes, e.g., MDM-2 and cyclin D. In addition to these, excessive growth factor signaling can be caused by the over expression of growth factors. Together with these gain-of-function mutations, growth factor receptors or downstream signal-transducing molecules can cause cell transformation by overriding the G1 checkpoint. In contrast, few cancers have disrupted G2 cell cycle arrest checkpoints. Thus, the G2 checkpoint is usually retained in cancer cells with the impaired G1 checkpoint.

If the G2 checkpoint could be selectively disrupted, cancer cells with an impaired G1 checkpoint would become more sensitive to DNA-damaging treatment, as compared to normal cells (with intact G1), since progression through G1 and G2 without repairing such damage induces apoptosis.

The mechanism that promotes the cell cycle G2 arrest after DNA damage is conserved among species from yeast to human. In the presence of damaged DNA, Cdc2/Cyclin B kinase is kept inactive because of inhibitory phosphorylation of threonine-14 and tyrosine-15 residues on Cdc2 kinase. At the onset of mitosis, the dual phosphatase
5 Cdc25 kinase removes these inhibitory phosphates and thereby activates Cdc2/Cyclin B kinase.

In fission yeast, the protein kinase Chk1 is required for the cell cycle arrest in response to damaged DNA. Chk1 kinase acts downstream of several rad gene products and is modified by the phosphorylation upon DNA damage. The kinases Rad53 of budding yeast
10 and Cds1 of fission yeast are known to conduct signals from unreplicated DNA. It appears that there is some redundancy between Chk1 and Cds1 because elimination of both Chk1 and Cds1 was culminated in disruption of the G2 arrest induced by damaged DNA. Interestingly, both Chk1 and Cds1 phosphorylate Cdc25 kinase and promote Rad24 binding to Cdc25, which sequesters Cdc25 to cytosol and prevents Cdc2/Cyclin B activation. Therefore Cdc25
15 appears to be a common target of these kinases and presumably an indispensable factor in the G2 checkpoint.

In humans, both hChk1, a human homologue of fission yeast Chk1, and Chk2/HuCds1, a human homologue of the budding yeast Rad53 and fission yeast Cds1, phosphorylate Cdc25C at serine-216, a critical regulatory site, in response to DNA damage.
20 This phosphorylation creates a binding site for small acidic proteins 14-3-3s, human homologues of Rad24 and Rad25 of fission yeast (Lopez-Girona (1999) Nature 397:172-175). The regulatory role of this phosphorylation was clearly indicated by the fact that substitution of serine-216 to alanine on Cdc25C disrupted cell cycle G2 arrest in human cells (Peng (1997) Science 277:1501-1505).

SUMMARY

25 This invention provides nucleic acids and polypeptides which can be used to treat cell proliferative disorders, such as those associated with benign and malignant tumor cells. While the invention is not limited to any particular mechanisms, the polypeptides of the invention can function by inhibiting the G2 cell cycle arrest checkpoint. Thus, the

invention also provides compositions and methods for selectively sensitizing a cell with an impaired G1 cell cycle arrest checkpoint, e.g., a cancer cell, to a DNA damaging agent

The invention provides an isolated or recombinant polypeptide comprising the amino acid sequence: X₁ X₂ X₃ X₄ X₅ X₆ X₇ X₈ X₉ X₁₀ X₁₁, wherein X₁ is L, F, W, M, R, I, V, Y, K, or absent, X₂ is Y, F, A, W, S or T, X₃ is any amino acid, X₄ is any amino acid, X₅ is any amino acid, X₆ is S, A, N, H or P, X₇ is any amino acid, X₈ is any amino acid, X₉ is any amino acid or absent, X₁₀ is N, G, L, S, M, P, N, A or absent, and X₁₁ is L or absent, wherein the polypeptide when administered to or expressed in a cell disrupts the G2 cell cycle arrest checkpoint.

In alternative embodiments, for the isolated or recombinant polypeptide of the invention: X₁ is L, F, W, M, R or absent or X₁ is L, F or W; X₂ is Y, F, A; X₃ is R, T, S, H, D, G, A, L, K, A, N, Q or P, or, X₃ is R, T, S, H, D, G, A or L, or, X₃ is R, T, S or H; X₄ is S, T, G, A, L, R, I, M, V, P, or, X₄ is S, T, G, A, L, R, or, X₄ is S; X₅ is P, A, G, S or T, or, X₅ is P; X₆ is S, N, H, P, A, G or T, or, X₆ is S, N or H, or, X₆ is S; X₇ is M, F, Y, D, E, N, Q, H, G, I, L, V, A, P, N or W, or, X₇ is M, F, Y, D, E, N, Q or H, or, X₇ is M, F, Y, Q or H; X₈ is P, F, Y, W, L, G, M, D, E, N, Q, H, I, V, A or P, or, X₈ is P, F, Y or W, or, X₈ is Y; X₉ is E, G, L, S, M, P, N, D, A, T, P or absent; X₁₀ is absent; X₁₁ is absent.

In one embodiment, the invention provides a polypeptide wherein X₂ is Y, X₅ is P, and X₁₀ is N. In one embodiment, the invention provides a polypeptide wherein X₃ is R, X₈ is P, and X₁₁ is L. In one embodiment, the invention provides a polypeptide wherein X₄ is S, X₅ is P, X₆ is S, X₉ is E, X₁₀ is N and X₁₁ is L.

In alternative embodiments, the invention provides an isolated or recombinant polypeptide wherein the amino acid sequence comprises Y G G P G G G G N; R Y S L P P E L S N M; L A R S A S M P E A L; L Y R S P S M P E N L; L Y R S P A M P E N L; W Y R S P S F Y E N L; W Y R S P S Y Y E N L; or, W Y R S P S Y Y.

In alternative embodiments, the invention provides an isolated or recombinant polypeptide wherein the amino acid sequence comprises L Y R S P S Y P E N L, L Y R S P S Y F E N L, L Y R S P S Y Y E N L, or L Y R S P S Y W E N L.

In alternative embodiments, the invention provides an isolated or recombinant polypeptide wherein the amino acid sequence comprises L Y R S P S N P E N L, L Y R S P S N F E N L, L Y R S P S N Y E N L, or L Y R S P S N W E N L.

In alternative embodiments, the invention provides an isolated or recombinant polypeptide wherein the amino acid sequence comprises L Y R S P S H P E N L, L Y R S P S H F E N L, L Y R S P S H Y E N L, L Y R S P S H W E N L, L Y S S P S M P E N L, L Y S S P S M F E N L, L Y S S P S M Y E N L, L Y S S P S M W E N L, L Y S S P S F P E N L, L Y S S P S F P E N L, L Y S S P S F F E N L, L Y S S P S F Y E N L, L Y S S P S F W E N L, L Y S S P S Y P E N L, L Y S S P S Y F E N L, L Y S S P S Y Y E N L, or L Y S S P S Y W E N L.

In alternative embodiments, the invention provides an isolated or recombinant polypeptide wherein the amino acid sequence comprises L Y S S P S Q P E N L, L Y S S P S Q W E N L, L Y S S P S H P E N L, L Y S S P S H F E N L, L Y S S P S H Y E N L, L Y S S P S H W E N L, L Y T S P S M P E N L, L Y T S P S M F E N L, L Y T S P S M Y E N L, L Y T S P S M W E N L, L Y T S P S F P E N L, L Y T S P S F F E N L, L Y T S P S F Y E N L, L Y T S P S F W E N L, L Y T S P S Y P E N L, L Y T S P S Y F E N L, L Y T S P S Y Y E N L, or L Y T S P S Y W E N L.

In alternative embodiments, the invention provides an isolated or recombinant polypeptide wherein the amino acid sequence comprises L Y T S P S N P E N L, L Y T S P S N F E N L, L Y T S P S N Y E N L or L Y T S P S N W E N L.

In alternative embodiments, the invention provides an isolated or recombinant polypeptide wherein the amino acid sequence comprises L Y T S P S H P E N L, L Y T S P S H F E N L, L Y T S P S H Y E N L or L Y T S P S H W E N L.

In alternative embodiments, the invention provides an isolated or recombinant polypeptide wherein the amino acid sequence comprises L Y H S P S Y P E N L, L Y H S P S Y F E N L, L Y H S P S Y Y E N L or L Y H S P S Y W E N L.

In alternative embodiments, the invention provides an isolated or recombinant polypeptide wherein the amino acid sequence comprises L F T S P S Y P E N L, L F T S P S Y F E N L, L F T S P S Y Y E N L or L F T S P S Y W E N L.

In alternative embodiments, the invention provides an isolated or recombinant polypeptide wherein the amino acid sequence comprises F Y S S P S H P E N L, F Y S S P S H F E N L, F Y S S P S H Y E N L, F Y S S P S H W E N L, F Y T S P S M P E N L, F Y T S P S M F E N L, F Y T S P S M Y E N L, F Y T S P S M W E N L, F Y T S P S F P

ENL, FYTSPSFFENL, FYTSPSFYENL, FYTSPSFWENL, FYTS
PSYPENL, FYTSPSYFENL, FYTSPSYYENL or FYTSPSYWENL.

In alternative embodiments, the invention provides an isolated or recombinant
polypeptide wherein the amino acid sequence comprises W Y R S P S M P E N L, W Y R S
5 P S M F E N L, W Y R S P S M Y E N L, W Y R S P S M W E N L, W Y R S P S F P E N
L, W Y R S P S F F E N L, W Y R S P S F Y E N L, W Y R S P S F W E N L, W Y R S P
S Y P E N L, W Y R S P S Y F E N L, W Y R S P S Y Y E N L or
W Y R S P S Y W E N L.

In alternative embodiments, the invention provides an isolated or recombinant
10 polypeptide wherein the amino acid sequence comprises W Y T S P S M P E N L, W Y T S
P S M F E N L, W Y T S P S M Y E N L, W Y T S P S M W E N L, W Y T S P S F P E N
L, W Y T S P S F F E N L, W Y T S P S F Y E N L, W Y T S P S F W E N L, W Y T S P
S Y P E N L, W Y T S P S Y F E N L, W Y T S P S Y Y E N L or
W Y T S P S Y W E N L.

15 In alternative embodiments, the invention provides an isolated or recombinant
polypeptide wherein the amino acid sequence comprises W Y T S P S H P E N L, W Y T S
P S H F E N L, W Y T S P S H Y E N L or W Y T S P S H W E N L.

In alternative embodiments, the invention provides an isolated or recombinant
polypeptide wherein the amino acid sequence comprises L K R S P S M P E N L, L Y I S P
20 S M P E N L or L Y R S P S M V E N L.

In one embodiment, the invention provides an isolated or recombinant
polypeptide wherein the polypeptide when administered to or expressed in a cell disrupts the
G2 cell cycle arrest checkpoint, wherein the cell is a mammalian cell. The cell can be a
human cell, a yeast cell, an insect cell, a bacterial cell, a plant cell, and the like.

25 In one embodiment, the invention provides an isolated or recombinant
polypeptide further comprising a cell membrane permeant. The cell membrane permeant can
comprise a polypeptide, such as a TAT protein transduction domain, e.g., comprising a
sequence Y G R K K R R Q R R R. Alternatively, the cell membrane permeant can comprise
a lipid, such as a liposome.

30 The invention provides a chimeric polypeptide comprising a first domain
comprising a polypeptide of the invention and a second domain comprising a cell membrane

permeant, wherein the polypeptide when administered to or expressed in a cell disrupts the G2 cell cycle arrest checkpoint. The chimeric polypeptide can be a recombinant fusion protein.

5 The invention provides an isolated or recombinant nucleic acid encoding a polypeptide or a chimeric polypeptide of the invention, wherein the polypeptide, when administered to or expressed in a cell, disrupts the G2 cell cycle arrest checkpoint.

The invention provides an expression vector comprising a nucleic acid encoding a polypeptide or a chimeric polypeptide of the invention, wherein the polypeptide, when administered to or expressed in a cell, disrupts the G2 cell cycle arrest checkpoint.

10 The invention provides a cell comprising a nucleic acid or an expression vector of the invention. The cell can be a bacterial, a yeast, an insect, a plant, or a mammalian cell.

15 The invention provides a pharmaceutical composition comprising a polypeptide of the invention, a nucleic acid of the invention, an expression vector of the invention, or a cell of the invention; and, a pharmaceutically acceptable excipient. In one embodiment, the pharmaceutical composition can comprise a liposome.

20 The invention provides a method for inhibiting the activity of a Chk1 kinase or a Chk2 kinase comprising contacting the kinase with a polypeptide of the invention or a pharmaceutical composition of the invention, in an amount sufficient to inhibit the activity of the Chk1 or Chk2 kinase.

25 The invention provides a method for disrupting a cell G2 cell cycle arrest checkpoint comprising contacting the cell with a polypeptide of the invention or a pharmaceutical composition of the invention in an amount sufficient to disrupt the G2 cell cycle arrest checkpoint. In alternative embodiments the cell is a mammalian cell, a human cell or a cancer cell.

30 The invention provides a method for sensitizing a cell to a DNA damaging agent comprising contacting the cell with a polypeptide of the invention or a pharmaceutical composition of the invention in an amount sufficient to disrupt the G2 cell cycle arrest checkpoint, thereby sensitizing the cell to the DNA damaging agent. In alternative embodiments the cell is a mammalian cell, a human cell or a cancer cell. The cancer cell can have an impaired G1 cell cycle arrest checkpoint.

The invention provides a method for selectively sensitizing a cell with an impaired G1 cell cycle arrest checkpoint to a DNA damaging agent comprising contacting the cell with a polypeptide of the invention or a pharmaceutical composition of the invention, in an amount sufficient to disrupt the G2 cell cycle arrest checkpoint, thereby sensitizing the cell to the DNA damaging agent. In alternative embodiments the cell is a mammalian cell, a human cell or a cancer cell.

The invention provides a method for inducing apoptosis in a cell in an individual comprising administering a polypeptide of the invention or a pharmaceutical composition of the invention, in an amount sufficient to disrupt the G2 cell cycle arrest checkpoint in the cancer cell, thereby sensitizing the cancer cell to a DNA damaging agent, and administering a DNA damaging agent. In alternative embodiments the cell is a mammalian cell, a human cell or a cancer cell. The cancer cell can have an impaired G1 cell cycle arrest checkpoint. The DNA damaging agent can be 5-fluorouracil (5-FU), rebeccamycin, adriamycin, bleomycin, cisplatin, hyperthermia, UV irradiation or gamma-irradiation.

The invention provides a method for screening for compounds capable of modulating the activity of a Chk1 kinase or a Chk2 kinase comprising the following steps: (a) providing a test compound; (b) providing a Chk1 kinase or a Chk2 kinase; (c) providing a polypeptide of the invention, wherein the polypeptide binds to the Chk1 kinase or the Chk2 kinase; and, (d) contacting the test compound with the kinase and the polypeptide and measuring the ability of the test compound to prevent binding of the polypeptide to the kinase.

The invention provides a method for screening for compounds capable of modulating the activity of a Chk1 kinase or a Chk2 kinase comprising the following steps: (a) providing a test compound; (b) providing a Chk1 kinase or a Chk2 kinase; (c) providing a polypeptide of the invention, wherein the polypeptide is phosphorylated by the Chk1 kinase or the Chk2 kinase; and, (d) contacting the test compound with the kinase and the polypeptide and measuring the ability of the test compound to inhibit or abrogate phosphorylation of the polypeptide by the kinase. The method can further comprising providing a full length human Cdc25C. In one embodiment of the method, the polypeptide of step (c) comprises amino acid residue serine 216 of human Cdc25C, such as comprising

from about amino acid residue 200 to about amino acid residue 250 of human Cdc25C. In one embodiment of the method, the polypeptide of step (c) further comprises glutathione-S-transferase.

In one embodiment of the methods of the invention, including the screening methods, the polypeptide of the invention is immobilized.

The invention provides a method for screening for compounds capable of specifically inhibiting the G2 cell cycle checkpoint comprising the following steps: (a) providing a test compound and a polypeptide of the invention; (b) providing a G1 checkpoint impaired cell; (c) contacting the cell of step (b) with the test compound or the polypeptide of step (a) plus a DNA damaging treatment, such as 5-fluorouracil (5-FU), rebeccamycin, adriamycin, bleomycin, cisplatin, hyperthermia, UV irradiation or gamma-irradiation, or, or an M phase checkpoint activator; and, (d) measuring the amount of DNA in the cells after the contacting of step (c) to determine if the test compound has inhibited the G2 cell cycle checkpoint, wherein the polypeptide of step (a) acts as a G2-checkpoint-inhibiting positive control. In alternative embodiments the cell is a mammalian cell, a human cell or a cancer cell. In one embodiment, the amount of DNA is measured using propidium iodide by, e.g., a FACS analysis, or equivalent. In one embodiment, the amount of DNA is measured after about 10 to about 72 hours after the contacting of step (c).

In one embodiment, the method comprises contacting the cell of step (b) with an M phase checkpoint activator alone (as a substitute for a DNA damaging agent) and the test compound or the polypeptide of step (a), wherein a test compound that has not inhibited or abrogated the arrest at the M phase checkpoint of the cell cycle after contacting the cell with an M phase activator is a specific inhibitor of the G2 cell cycle checkpoint (because it did not affect M phase checkpoint or it was not a non-specific phenomenon). In one embodiment, the M phase checkpoint activator is colchicine or nocodazole.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

All publications, patents, patent applications, GenBank sequences and ATCC deposits, cited herein are hereby expressly incorporated by reference for all purposes.

DESCRIPTION OF DRAWINGS

Figure 1 shows chimeric peptides used in and results of experiments demonstrating that TAT-S216A and TAT-S216 peptides inhibit hChk1 and Chk2/HuCds1 kinase activity *in vitro*, as described in Example 1, below. Figure 1A shows a schematic diagram of the fusion/chimeric peptides TAT-control, TAT-S216A and TAT-S216. Figure 1B shows SDS-PAGE autoradiograms demonstrating the results of *in vitro* Cdc25C phosphorylation assays using TAT-S216A and TAT-S216 peptides to inhibit purified hChk1 activity; amino acid residues 200 to 256 of Cdc25C (SEQ ID NO:1) were used as a substrate at a concentration of 1 μ M. Figure 1C shows SDS-PAGE autoradiograms demonstrating the results of *in vitro* Cdc25C phosphorylation assays using TAT-S216A peptide to inhibit purified hChk1 and Chk2/HuCds1 activity; amino acid residues 211 to 220 of Cdc25C (SEQ ID NO:1) were used as a substrate at a concentration of 10 μ M.

Figure 2 the results of experiments demonstrating that TAT-S216A and TAT-S216 peptides can abrogate DNA damage-induced G2 arrest in Jurkat cells. Figure 2A shows the results of a FACS analysis of Jurkat cells treated with bleomycin (10 μ g/ml) and TAT-S216A and TAT-S216 peptides (10 μ M each). Figure 2B shows the results of an SDS-PAGE of cell lysates from a histone H1 kinase analysis; lysates were prepared from cells treated with the indicated reagent for six hours. Figure 2C shows the results a FACS analysis of colchicines- (5 μ g/ml) and peptide- (10 μ M each) treated cells; Jurkat cells were treated for 20 hours.

Figure 3 shows the results of experiments demonstrating that TAT-S216A and TAT-S216 peptides can specifically sensitize cancer cells to bleomycin, but not colchicine. Figure 3A shows the results of trypan blue dye exclusion analysis of Jurkat cells treated with bleomycin with or without the TAT-S216A and TAT-S216 peptides. Figure 3B shows the results of trypan blue dye exclusion (survival) analysis of Jurkat cells treated with colchicine with or without the TAT-S216A and TAT-S216 peptides. Figure 3C shows the results of trypan blue dye exclusion (survival) analysis of PHA blasts treated with bleomycin with or without the TAT-S216A and TAT-S216 peptides. Figure 3D shows the results of FACS analysis PHA blasts treated with bleomycin with or without the TAT-S216A and TAT-S216 peptides (vertical axis is DNA content indicated by propidium iodide staining).

Figure 4 shows the results of experiments demonstrating that TAT-S216A and TAT-S216 peptides can sensitize cancer cells to bleomycin. Figure 4A shows the results of X-TT analysis of PANC1 cells treated with bleomycin with or without the TAT-S216A and TAT-S216 peptides. Figure 4B shows the results of X-TT analysis of MIA PaCa2 cells treated with bleomycin with or without the TAT-S216A and TAT-S216 peptides.

Figure 5 shows a schematic 3-dimensional structure of human Chk2 interacting with exemplary G2-abrogating peptides of the invention, as described in Example 2, below.

Figure 6 shows the results of FACS analysis of the amount of DNA in cells to determine the number of cells in one of the four cell cycle phases after incubating these cells with bleomycin and exemplary peptides of the invention, as described in Example 3, below.

Figure 7 shows the results of FACS analysis of the amount of DNA in cells to determine the number of cells in one of the four cell cycle phases after incubating these cells with colchicine and exemplary peptides of the invention, as described in Example 3, below.

Figure 8 shows the sequences of peptides used in experiments described in Example 4, below.

Figure 9 shows a summary of results of experiments as described in Example 4, below.

Figure 10 shows the results of experiments demonstrating that a peptide of the invention (as a S216-containing fusion protein) administered to an animal *in vivo* effectively sensitized cancer cells to a DNA damaging agent.

Figure 11 shows the results of experiments demonstrating that a peptide of the invention (as a R-II-containing fusion protein) administered to an animal *in vivo* effectively sensitized cancer cells to a DNA damaging agent.

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

The genes and polypeptides of the invention provide a novel means to treat cell proliferative disorders, including, e.g., to stop the growth of, or kill, cancer cells. While the invention is not limited by any particular mechanism of action, administration of the polypeptides of the invention will delay or abrogate G2 cell cycle arrest checkpoint in cells.

The genes and polypeptides of the invention can also be used to inhibit Chk1 and/or Chk2/Cds1 kinase activity. Inhibition of Chk1 and/or Chk2/Cds1 kinase may be the mechanism by which the G2 checkpoint is inhibited. The invention also provides methods for selectively sensitizing G1 checkpoint impaired cancer cells to DNA damaging agents and treatments. Also provided are methods for screening for compounds able to interact with, e.g., inhibit, enzymes involved in the G2 cell cycle arrest checkpoint, such as Chk1 and/or Chk2/Cds1 kinases. Thus, the invention provides methods to screen for compounds that inhibit or abrogate cell cycle G2 checkpoint.

The invention for the first time describes amino acid peptide motifs in the human Cdc25C (hCdc25C) polypeptide (SEQ ID NO:1) that are the substrate motifs for human Chk1 (hChk1) (SEQ ID NO:3) and human Chk2/ human Cds1 (Chk2/HuCds1) (SEQ ID NO:4) kinase activity. The kinase-inhibitory polypeptides and nucleic acids of the invention are modeled on these hCdc25C peptide motifs. Wild-type hCdc25C is phosphorylated by hChk1 (SEQ ID NO:3) and Chk2/HuCds1 (SEQ ID NO:4).

Phosphorylation of Cdc25C is necessary for the cell's arrest at G2 checkpoint. Thus, the polypeptides and peptides of the invention, by inhibiting the phosphorylation of Cdc25C (by enzymes which probably include Chk1 and Chk2/HuCds1), can inhibit or abrogate the cell's G2 checkpoint capability. The lack of an effective G2 checkpoint after DNA damage becomes fatal to the cell (see, e.g., Maity (1994) Radiother. Oncol. 31:1-13). If a cell progresses through G2 without sufficient repair of DNA damage it becomes apoptotic. Thus, the compositions of the invention can be used to sensitize cells, such as tumor cells, to DNA damaging agents. In fact, as discussed below, the compositions of the invention can sensitize cancer cells to the apoptotic effects of DNA-damaging agents with little or no cytotoxic effect on normal cells.

Example 1, below, describes the synthesis and use of two exemplary polypeptides of the invention. Two peptides corresponding to amino acids 211 to 221 of human Cdc25C (SEQ ID NO:1) fused with a part of HIV-1-TAT (SEQ ID NO:5). These peptides were demonstrated to inhibit hChk1 kinase (SEQ ID NO:3) and Chk2/HuCds1 kinase (SEQ ID NO:4) activity *in vitro* and to specifically abrogate the G2 checkpoint *in vivo*. These peptides sensitized p53-defective cancer cell lines to the apoptotic effects of DNA-damaging agents without obvious cytotoxic effect on normal cells. These results

clearly demonstrate that the polypeptides comprising the motifs of the invention can be used to specifically inhibit or abrogate the cell cycle G2 checkpoint. These results demonstrate that the compositions of the invention can be used to screen for compositions that inhibit Chk1 or Chk2 kinase activity. These results also demonstrate that the compositions of the invention can be used for cancer therapy. While the invention is not limited by any particular mechanism of action, the polypeptides and peptides of the invention can be used to target and inhibit hChk1 (SEQ ID NO:3) and Chk2/HuCds1 (SEQ ID NO:4) kinases.

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term “cell membrane permeant” as used herein means any composition which, when associated with a peptide or polypeptide of the invention, or a nucleic acid of the invention, causes, or assists in, the internalization of the composition into a cell. The association can be covalent (e.g., a linking reagent, or, as a fusion protein) or non-covalent (e.g., as with liposomes). For example, in one embodiment, a cell membrane permeant domain is linked to a peptide or polypeptide of the invention as a fusion protein domain, e.g., a TAT protein transduction domain (see, e.g., Vives (1997) J. Biol. Chem. 272:16010-16017). Other cell membrane permeant domains include, e.g., the PreS2- and S-domain of the hepatitis-B virus surface antigens, see, e.g., Oess (2000) Gene Ther. 7:750-758.

The term “human Cdc25C” or “hCdc25C” as used herein means, depending on the context, the human Cdc25C polypeptide (SEQ ID NO:1) or the human Cdc25C polypeptide (SEQ ID NO:1) message (cDNA) (SEQ ID NO:2) or gene (see, e.g., Peng (1997) Science 277:1501-1505). The term also includes all functional variations of hCdc25C, including, e.g., allelic variations, functional mutations, variations with additions, deletions, substitutions that retain functional activity. A Cdc25C polypeptide that has functional activity has the same activity as wild type Cdc25C, i.e., when appropriately phosphorylated, it can act in concert with other cell cycle control polypeptides to arrest cell growth at G2 under the proper conditions, e.g., under conditions in which sufficient DNA damage has incurred to induce apoptosis if the cell passes through the G2 checkpoint.

The terms "DNA damaging treatment" or "DNA damaging agent" include any treatments or agents that will cause DNA damage to a cell, including a drug, a radiation, an environmental shock, and the like, including, e.g., hyperthermia, UV radiation or gamma-radiation, in addition to the known DNA damaging drugs, e.g., 5-fluorouracil (5-FU), rebeccamycin, adriamycin, bleomycin, cisplatin and the like.

The term "disrupt the cell cycle G2 checkpoint" or "inhibit the cell cycle G2 checkpoint" means the ability of a peptide or polypeptide of the invention to inhibit (including abrogate) a Chk1 kinase and/or Chk2 kinase activity, e.g., a mammalian kinase, such as a human Chk1 (hChk1) kinase (SEQ ID NO:3) (see, e.g., Yin (2000) Mol. Pharmacol. 57:453-459) or a human Chk2/ human Cds1 kinase (Chk2/HuCds1) (SEQ ID NO:4) (see, e.g., Hirao (2000) Science 287:1824-1827), or, to disrupt (including abrogate) the ability of a cell to arrest growth at the G2 checkpoint under appropriate conditions, e.g., where conditions in the cell otherwise would cause G2 cell cycle arrest, such as the accumulation of DNA damage by, e.g., some anti-tumor agents.

The ability of a peptide or polypeptide of the invention to modulate or inhibit a Chk1 kinase and/or a Chk2 kinase activity can be easily tested *in vitro* or *in vivo* as, for example, in the assays, or variations thereof, described in Example 1, below. A peptide or polypeptide is considered an effective inhibitor if, e.g., it binds the kinase to inhibit or abrogate kinase activity. Alternatively, a peptide or polypeptide is also considered an effective inhibitor of kinase activity if it acts as a phosphorylation substrate and prevents phosphorylation of natural substrate, e.g., wild type Cdc25C, thereby disrupt the ability of a cell to arrest growth at the G2 checkpoint under appropriate conditions.

The ability of exemplary peptides or polypeptides of the invention to disrupt the ability of a cell to arrest growth at the G2 checkpoint, i.e., to act in concert with other cell cycle control polypeptides to arrest cell growth at G2 under the proper conditions, e.g., under conditions in which sufficient DNA damage has incurred to induce apoptosis if the cell passes through the G2 checkpoint can be easily tested *in vivo*, e.g., cell culture, is demonstrated in Example 1, below

The term "expression cassette" as used herein refers to a nucleotide sequence which is capable of affecting expression of a structural gene (i.e., a protein coding sequence) in a host compatible with such sequences. Expression cassettes include at least a promoter

operably linked with the polypeptide coding sequence; and, optionally, with other sequences, e.g., transcription termination signals. Additional factors necessary or helpful in effecting expression may also be used, e.g., enhancers. "Operably linked" as used herein refers to linkage of a promoter upstream from a DNA sequence such that the promoter mediates transcription of the DNA sequence. Thus, expression cassettes also include plasmids, expression vectors, recombinant viruses, any form of recombinant "naked DNA" vector, and the like. A "vector" comprises a nucleic acid which can infect, transfect, transiently or permanently transduce a cell. It will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes (e.g., a cell membrane, a viral lipid envelope, etc.). Vectors include, but are not limited to replicons (e.g., RNA replicons, bacteriophages) to which fragments of DNA may be attached and become replicated. Vectors thus include, but are not limited to RNA, autonomous self-replicating circular or linear DNA or RNA (e.g., plasmids, viruses, and the like, see, e.g., U.S. Patent No. 5,217,879), and includes both the expression and nonexpression plasmids. Where a recombinant microorganism or cell culture is described as hosting an "expression vector" this includes both extrachromosomal circular and linear DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

The term "chemically linked" refers to any chemical bonding of two moieties, e.g., as in one embodiment of the invention, a polypeptide comprising at least two peptide motifs of the invention. Such chemical linking includes the peptide bonding of a recombinantly or *in vivo* generated fusion protein.

The term "chimeric protein" or "fusion protein" refers to a composition comprising at least one polypeptide or peptide domain or motif which is associated with a second polypeptide or peptide domain or motif. For example, in one embodiment, the invention provides an isolated or recombinant nucleic acid molecule encoding a fusion protein comprising at least two domains, wherein the first domain comprises one kinase-inhibiting or G2-checkpoint inhibiting motif and the second domain comprising a second motif with the same or similar activity (for example, on motif may have a high binding

affinity for the kinase, whilst the second motif has high kinase inhibitory activity).

Additional domains can comprise a polypeptide, peptide, polysaccharide, or the like. The “fusion” can be an association generated by a peptide bond, a chemical linking, a charge interaction (*e.g.*, electrostatic attractions, such as salt bridges, H-bonding, etc.) or the like. If the polypeptides are recombinant, the “fusion protein” can be translated from a common message. Alternatively, the compositions of the domains can be linked by any chemical or electrostatic means. The chimeric molecules of the invention can also include additional sequences, *e.g.*, linkers, epitope tags, enzyme cleavage recognition sequences, signal sequences, secretion signals, and the like. Alternatively, a peptide can be linked to a carrier simply to facilitate manipulation or identification/ location of the peptide.

The term “G2 checkpoint inhibitory activity” as used herein means any amount of inhibition of the G2 checkpoint.

The term “isolated” as used herein, when referring to a molecule or composition, such as, *e.g.*, a nucleic acid or polypeptide of the invention, means that the molecule or composition is separated from at least one other compound, such as a protein, other nucleic acids (*e.g.*, RNAs), or other contaminants with which it is associated *in vivo* or in its naturally occurring state. Thus, a nucleic acid or polypeptide is considered isolated when it has been isolated from any other component with which it is naturally associated, *e.g.*, cell membrane, as in a cell extract. An isolated composition can, however, also be substantially pure. An isolated composition can be in a homogeneous state and can be in a dry or an aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemistry techniques such as polyacrylamide gel electrophoresis (SDS-PAGE) or high performance liquid chromatography (HPLC). Thus, the isolated compositions of this invention do not contain materials normally associated with their *in situ* environment. Even where a protein has been isolated to a homogenous or dominant band, there can be trace contaminants which co-purify with the desired protein.

The terms “polypeptide,” “protein,” and “peptide” include compositions of the invention that also include “analogs,” or “conservative variants” and “mimetics” or “peptidomimetics” with structures and activity that substantially correspond to the polypeptide from which the variant was derived, including, *e.g.*, variations of the peptides

and polypeptides of the invention which can either inhibit a mammalian Chk1 and/or Chk2 kinase, or, inhibit a mammalian G2 checkpoint.

The term "pharmaceutical composition" refers to a composition suitable for pharmaceutical use, e.g., as an anti-cancer agent, in a subject. The pharmaceutical compositions of this invention are formulations that comprise a pharmacologically effective amount of a composition comprising, e.g., a peptide, polypeptide, nucleic acid, vector, or cell of the invention, and a pharmaceutically acceptable carrier.

The term "promoter" is an array of nucleic acid control sequences which direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter which is active under most environmental and developmental conditions. An "inducible" promoter is a promoter which is under environmental or developmental regulation. A "tissue specific" promoter is active in certain tissue types of an organism, but not in other tissue types from the same organism. The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

The term "recombinant" refers to a polynucleotide synthesized or otherwise manipulated *in vitro* (e.g., "recombinant polynucleotide"), to methods of using recombinant polynucleotides to produce gene products in cells or other biological systems, or to a polypeptide ("recombinant protein") encoded by a recombinant polynucleotide. For example, recombinant peptides or polypeptides or nucleic acids can be used to practice the methods of the invention. "Recombinant means" also encompass the ligation of nucleic acids having various coding regions or domains or promoter sequences from different sources into an expression cassette or vector for expression of, e.g., inducible or constitutive expression of polypeptide coding sequences in the vectors used to practice this invention.

Nucleic Acids and Expression Vectors

This invention provides novel nucleic acids, including expression vectors, for use in the treatment of uncontrolled cell growth, such as cancer, and means to make and express those nucleic acids. As the genes and vectors of the invention can be made and expressed *in vitro* or *in vivo*, the invention provides for a variety of means of making and expressing these genes and vectors. One of skill will recognize that desired levels of expression of the polypeptides of the invention can be obtained by modulating the expression or activity of the genes and nucleic acids (e.g., promoters) within the vectors of the invention. Any of the known methods described for increasing or decreasing expression or activity, including tissue-specific expression, can be used for this invention. The invention can be practiced in conjunction with any method or protocol known in the art, which are well described in the scientific and patent literature.

General Techniques

The nucleic acid sequences of the invention and other nucleic acids used to practice this invention, whether RNA, cDNA, genomic DNA, vectors, viruses or hybrids thereof, may be isolated from a variety of sources, genetically engineered, amplified, and/or expressed recombinantly. Any recombinant expression system can be used, including, in addition to bacterial cells, e.g., mammalian, yeast, insect or plant cell expression systems.

Alternatively, these nucleic acids can be synthesized *in vitro* by well-known chemical synthesis techniques, as described in, e.g., Carruthers (1982) Cold Spring Harbor Symp. Quant. Biol. 47:411-418; Adams (1983) J. Am. Chem. Soc. 105:661; Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med. 19:373-380; Blommers (1994) Biochemistry 33:7886-7896; Narang (1979) Meth. Enzymol. 68:90; Brown (1979) Meth. Enzymol. 68:109; Beaucage (1981) Tetra. Lett. 22:1859; U.S. Patent No. 4,458,066. Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Techniques for the manipulation of nucleic acids, such as, e.g., generating mutations in sequences, subcloning, labeling probes, sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook, ed.,

MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993).

Nucleic acids, vectors, capsids, polypeptides, and the like can be analyzed and quantified by any of a number of general means well known to those of skill in the art. These include, e.g., analytical biochemical methods such as NMR, spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography, various immunological methods, e.g. fluid or gel precipitin reactions, immunodiffusion, immuno-electrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, Southern analysis, Northern analysis, dot-blot analysis, gel electrophoresis (e.g., SDS-PAGE), nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography. Amplification methods include, e.g., polymerase chain reaction, PCR (PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, ed. Innis, Academic Press, N.Y. (1990) and PCR STRATEGIES (1995), ed. Innis, Academic Press, Inc., N.Y., ligase chain reaction (LCR) (see, e.g., Wu (1989) Genomics 4:560; Landegren (1988) Science 241:1077; Barringer (1990) Gene 89:117); transcription amplification (see, e.g., Kwoh (1989) Proc. Natl. Acad. Sci. USA 86:1173); and, self-sustained sequence replication (see, e.g., Guatelli (1990) Proc. Natl. Acad. Sci. USA 87:1874); Q Beta replicase amplification (see, e.g., Smith (1997) J. Clin. Microbiol. 35:1477-1491), automated Q-beta replicase amplification assay (see, e.g., Burg (1996) Mol. Cell. Probes 10:257-271) and other RNA polymerase mediated techniques (e.g., NASBA, Cangene, Mississauga, Ontario); see also Berger (1987) Methods Enzymol. 152:307-316; Sambrook; Ausubel; U.S. Patent Nos. 4,683,195 and 4,683,202; Sooknanan (1995) Biotechnology 13:563-564.

Once amplified, the libraries can be cloned, if desired, into any of a variety of vectors using routine molecular biological methods; methods for cloning *in vitro* amplified nucleic acids are described, e.g., U.S. Pat. No. 5,426,039. To facilitate cloning of amplified sequences, restriction enzyme sites can be "built into" the PCR primer pair.

The invention provides libraries of expression vectors encoding polypeptides and peptides of the invention. These nucleic acids may be introduced into a genome or into the cytoplasm or a nucleus of a cell and expressed by a variety of conventional techniques, well described in the scientific and patent literature. See, e.g., Roberts (1987) Nature 328:731; Schneider (1995) Protein Expr. Purif. 6435:10; Sambrook, Tijssen or Ausubel. The vectors can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries, or prepared by synthetic or recombinant methods. For example, the nucleic acids of the invention can be expressed in expression cassettes, vectors or viruses which are stably or transiently expressed in cells (e.g., episomal expression systems). Selection markers can be incorporated into expression cassettes and vectors to confer a selectable phenotype on transformed cells and sequences. For example, selection markers can code for episomal maintenance and replication such that integration into the host genome is not required.

In one embodiment, the nucleic acids of the invention are administered *in vivo* for *in situ* expression of the peptides or polypeptides of the invention. The nucleic acids can be administered as "naked DNA" (see, e.g., U.S. Patent No. 5,580,859) or in the form of an expression vector, e.g., a recombinant virus. The nucleic acids can be administered by any route, including peri- or intra-tumorally, as described below. Vectors administered *in vivo* can be derived from viral genomes, including recombinantly modified enveloped or non-enveloped DNA and RNA viruses, preferably selected from baculoviridae, parvoviridae, picornaviridae, herpesviridae, poxviridae, adenoviridae, or picornaviridae. Chimeric vectors may also be employed which exploit advantageous merits of each of the parent vector properties (See e.g., Feng (1997) Nature Biotechnology 15:866-870). Such viral genomes may be modified by recombinant DNA techniques to include the nucleic acids of the invention; and may be further engineered to be replication deficient, conditionally replicating or replication competent. In alternative embodiments, vectors are derived from the adenoviral (e.g., replication incompetent vectors derived from the human adenovirus genome, see, e.g., U.S. Patent Nos. 6,096,718; 6,110,458; 6,113,913; 5,631,236); adeno-associated viral and retroviral genomes. Retroviral vectors can include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof;

see, e.g., U.S. Patent Nos. 6,117,681; 6,107,478; 5,658,775; 5,449,614; Buchscher (1992) J. Virol. 66:2731-2739; Johann (1992) J. Virol. 66:1635-1640). Adeno-associated virus (AAV)-based vectors can be used to transduce cells with target nucleic acids, e.g., in the *in vitro* production of nucleic acids and peptides, and in *in vivo* and *ex vivo* gene therapy procedures; see, e.g., U.S. Patent Nos. 6,110,456; 5,474,935; Okada (1996) Gene Ther. 3:957-964.

The peptides and polypeptides of the invention are derived from, or, based on, the structure of the kinase Cdc25C. The cDNA nucleic acid sequence for hCdc25C is

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1  caggaagact ctgagtccga cgtggccta cccagtcgga aggcagagct gcaatctagt
10      61 taactacctc cttcccta gatttcctt cattctgctc aagtcttcgc ctgtgtccga
      121 tccctatcta ctttctcc tctgtagca agcctcagac tccaggcttg agctaggttt
      181 tgttttctc ctggtgagaa ttcgaagacc atgtctacgg aactctctc atccacaaga
      241 gaggaaggaa gctctggctc aggaccagc ttaggtcta atcaaaggaa aatgttaaac
      301 ctgctctcgg agagagacac ttctttacc gtctgtccag atgtccctag aactccagtg
15      361 ggcaaatttc ttggtgattc tgcaaacctc agcattttgt ctggaggaac cccaaaatgt
      421 tgcctcgatc ttccaatct tagcagtggg gagataactg ccactcagct taccacttct
      481 gcagaccttg atgaaactgg tcacctggat tcttcaggac ttcaggaagt gcatttagct
      541 gggatgaatc atgaccagca cctaataaaa ttagcccag cacagcttct tttagcact
      601 ccgaatggtt tggacctgg ccatagaaag agagatgcaa tgtgtagttc atctgcaaat
20      661 aaagaaaatg acaatggaaa ctggtggac agtgaaatga aatatttggg cagtcccatt
      721 actactgttc caaaattgga taaaatcca aacctaggag aagaccaggc agaagagatt
      781 tcagatgaat taatggagtt ttccctgaaa gatcaagaag caaaggtgag cagaagtggc
      841 ctatatcgct ccccgctgat gccagagaac tgaacaggc caagactgaa gcaggtggaa
      901 aaattcaagg acaacacaat accagataaa gttaaaaaaa agtattttc tgccaagga
25      961 aagctcagga agggcttatg tttaagaag acagtctctc tgtgtgacat tactatcact
      1021 cagatgctgg aggaagattc taaccagggg cacctgattg gtgattttc caaggtatgt
      1081 gcgctgcaa ccggtgcagg gaaacaccaa gatctgaagt atgtcaacc agaaacagt
      1141 gctgccttac tgcggggaa gttccagggt ctgattgaga agttttatgt cattgattgt
      1201 cgctatccat atgagtatct gggaggacac atccagggag ccttaaactt atatagtcag
30      1261 gaagaactgt ttaactctt tctgaagaag cccatcgctc cttggacac ccagaagaga
      1321 ataatcatcg tgttccactg tgaattctcc tcagagaggg gccccgaat gtgccgctgt
      1381 ctgctgaag aggcaggtc tctgaaccag tatctgcat tgtactacc agagctatat
      1441 atcctaaag gcggctacag agacttctt ccagaatata tggaactgtg tgaaccacag
      1501 agctactgcc ctatgcatca tcaggaccac aagactgagt tgctgaggtg tcgaagccag
35      1561 agcaaagtgc aggaagggga gcggcagctg cgggagcaga tgccttctt ggtgaaggac

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1621 atgagcccat gataacattc cagccactgg ctgctaaca gtcacaaaa agacactgca
 1681 gaaacctga gcagaaagag gcctctgga tggccaaacc caagattatt aaaagatgc
 1741 tctgcaaacc aacaggctac caactgtat ccaggcctgg gaatggatta gggttcagca
 1801 gagctgaaag ctggtggcag agtctggag ctggctctat aaggcagcct tgagttgcat
 1861 agagatttgt attggttcag ggaactctgg cattccttt cccaactcct catgtcttct
 1921 cacaagccag ccaactcttt ctctctgggc ttggggctat gcaagagcgt tgtctacct
 1981 cttcttgtt attttcttc ttgtttccc cctctttct ttttaaaat ggaaaaataa
 2041 acactacaga atgag (SEQ ID NO:6)

The amino acid sequence of human hCdc25C is

MSTELFSSTREEGSSGSGSPFRSNQRKMLNLLLRDTSFTVCPD
 VPRTPVGKFLGDSANLSILSGGTPKCCLDLSNLSSGEITATQLTTSADLDETGHLDSS
 LQEVHLAGMNHQHLMKCSAQLLCSTPNGLDRGHRKRDAMCSSSANKENDNGNLVD
 SEMKYLGSPIITVPKLDKNPNLGEDQAEIISDELMEFSLKDQEAQVSRSGLYRSPSMP
 ENLNRPRLKQVEKFKDNTIPDKVKKKYFSGQGKLRKGLCLKKTVSLCDITITQMLEED
 SNQGHGIGDFSKVCALPTVSGKHQDLKYVNPETVAALLSGKFQGLIEKFYVIDCRYPY
 EYLGGHIQGALNLYSQEELFNFFLKPIVPLDTQKRIIVFHCFSSERGPRMCRCLR
 EEDRSLNQYPALYPELYILKGGYRDFPEYMELCPEQSYCPMHQDHKTELLRCRSQ
 SKVQEGERQLREQIALLVKDMSP (SEQ ID NO:1)

See also, e.g., GenBank Accession Nos. NP 001781 (protein) and NM
 001790 (nucleic acid, cDNA) and Sadhu (1990) Proc. Natl. Acad. Sci. U.S.A. 87:5139-5143.

Peptides and Polypeptides

The peptides and polypeptides of the invention can be administered to treat cell proliferative disorders, including, e.g., to stop the growth of, or kill, cancer cells. The peptides and polypeptides of the invention can be used to inhibit (e.g., delay) or abrogate G2 cell cycle arrest checkpoint in cells. The peptides and polypeptides of the invention can also be used to inhibit Chk1 and/or Chk2/Cds1 kinase activity.

While the peptides and polypeptides of the invention can be expressed recombinantly *in vivo* after administration of nucleic acids, as described above, they can also be administered directly, e.g., as a pharmaceutical composition.

Polypeptides and peptides of the invention can be isolated from natural sources, be synthetic, or be recombinantly generated polypeptides. Peptides and proteins can be recombinantly expressed *in vitro* or *in vivo*. The peptides and polypeptides of the invention can be made and isolated using any method known in the art. Polypeptide and

peptides of the invention can also be synthesized, whole or in part, using chemical methods well known in the art. See e.g., Caruthers (1980) Nucleic Acids Res. Symp. Ser. 215-223; Horn (1980) Nucleic Acids Res. Symp. Ser. 225-232; Banga, A.K., Therapeutic Peptides and Proteins, Formulation, Processing and Delivery Systems (1995) Technomic Publishing Co., Lancaster, PA. For example, peptide synthesis can be performed using various solid-phase techniques (see e.g., Roberge (1995) Science 269:202; Merrifield (1997) Methods Enzymol. 289:3-13) and automated synthesis may be achieved, e.g., using the ABI 431A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

The peptides and polypeptides of the invention, as defined above, include all “mimetic” and “peptidomimetic” forms. The terms “mimetic” and “peptidomimetic” refer to a synthetic chemical compound which has substantially the same structural and/or functional characteristics of the polypeptides of the invention. The mimetic can be either entirely composed of synthetic, non-natural analogues of amino acids, or, is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetic’s structure and/or activity. As with polypeptides of the invention which are conservative variants, routine experimentation will determine whether a mimetic is within the scope of the invention, i.e., that its structure and/or function is not substantially altered. Thus, a mimetic composition is within the scope of the invention if, when administered to or expressed in a cell, it disrupts the G2 cell cycle arrest checkpoint. A mimetic composition can also be within the scope of the invention if it can inhibit Chk1 and/or Chk2/Cds1 kinase activity, or, bind to the active site of either of these enzymes.

Polypeptide mimetic compositions can contain any combination of non-natural structural components, which are typically from three structural groups: a) residue linkage groups other than the natural amide bond (“peptide bond”) linkages; b) non-natural residues in place of naturally occurring amino acid residues; or c) residues which induce secondary structural mimicry, i.e., to induce or stabilize a secondary structure, e.g., a beta turn, gamma turn, beta sheet, alpha helix conformation, and the like. For example, a polypeptide can be characterized as a mimetic when all or some of its residues are joined by chemical means other than natural peptide bonds. Individual peptidomimetic residues can be

joined by peptide bonds, other chemical bonds or coupling means, such as, e.g., glutaraldehyde, N-hydroxysuccinimide esters, bifunctional maleimides, N,N'-dicyclohexylcarbodiimide (DCC) or N,N'-diisopropylcarbodiimide (DIC). Linking groups that can be an alternative to the traditional amide bond ("peptide bond") linkages include, e.g., ketomethylene (e.g., -C(=O)-CH₂- for -C(=O)-NH-), aminomethylene (CH₂-NH), ethylene, olefin (CH=CH), ether (CH₂-O), thioether (CH₂-S), tetrazole (CN₄-), thiazole, retroamide, thioamide, or ester (see, e.g., Spatola (1983) in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp 267-357, "Peptide Backbone Modifications," Marcell Dekker, NY).

A polypeptide can also be characterized as a mimetic by containing all or some non-natural residues in place of naturally occurring amino acid residues. Non-natural residues are well described in the scientific and patent literature; a few exemplary non-natural compositions useful as mimetics of natural amino acid residues and guidelines are described below. Mimetics of aromatic amino acids can be generated by replacing by, e.g., D- or L- naphylalanine; D- or L- phenylglycine; D- or L-2 thieneylalanine; D- or L-1, -2, 3-, or 4- pyreneylalanine; D- or L-3 thieneylalanine; D- or L-(2-pyridinyl)-alanine; D- or L-(3-pyridinyl)-alanine; D- or L-(2-pyrazinyl)-alanine; D- or L-(4-isopropyl)-phenylglycine; D-(trifluoromethyl)-phenylglycine; D-(trifluoromethyl)-phenylalanine; D-p-fluoro-phenylalanine; D- or L-p-biphenylphenylalanine; K- or L-p-methoxy-biphenylphenylalanine; D- or L-2-indole(alkyl)alanines; and, D- or L-alkylainines, where alkyl can be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, isopentyl, or a non-acidic amino acids. Aromatic rings of a non-natural amino acid include, e.g., thiazolyl, thiophenyl, pyrazolyl, benzimidazolyl, naphthyl, furanyl, pyrrolyl, and pyridyl aromatic rings.

Mimetics of acidic amino acids can be generated by substitution by, e.g., non-carboxylate amino acids while maintaining a negative charge; (phosphono)alanine; sulfated threonine. Carboxyl side groups (e.g., aspartyl or glutamyl) can also be selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as, e.g., 1-cyclohexyl-3(2-morpholinyl)-(4-ethyl) carbodiimide or 1-ethyl-3(4-azonia- 4,4- dimetholpentyl) carbodiimide. Aspartyl or glutamyl can also be converted to asparaginy and glutaminy residues by reaction with ammonium ions.

Mimetics of basic amino acids can be generated by substitution with, e.g., (in addition to lysine and arginine) the amino acids ornithine, citrulline, or (guanidino)-acetic acid, or (guanidino)alkyl-acetic acid, where alkyl is defined above. Nitrile derivative (e.g., containing the CN-moiety in place of COOH) can be substituted for asparagine or glutamine. Asparaginyll and glutaminyll residues can be deaminated to the corresponding aspartyl or glutamyl residues.

Arginine residue mimetics can be generated by reacting arginyl with, e.g., one or more conventional reagents, including, e.g., phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, or ninhydrin, preferably under alkaline conditions. Tyrosine residue mimetics can be generated by reacting tyrosyl with, e.g., aromatic diazonium compounds or tetranitromethane. N-acetylimidizol and tetranitromethane can be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Cysteine residue mimetics can be generated by reacting cysteinyl residues with, e.g., alpha-haloacetates such as 2-chloroacetic acid or chloroacetamide and corresponding amines; to give carboxymethyl or carboxyamidomethyl derivatives. Cysteine residue mimetics can also be generated by reacting cysteinyl residues with, e.g., bromo-trifluoroacetone, alpha-bromo-beta-(5-imidozoyl) propionic acid; chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide; methyl 2-pyridyl disulfide; p-chloromercuribenzoate; 2-chloromercuri-4-nitrophenol; or, chloro-7-nitrobenzo-oxa-1,3-diazole. Lysine mimetics can be generated (and amino terminal residues can be altered) by reacting lysinyl with, e.g., succinic or other carboxylic acid anhydrides. Lysine and other alpha-amino-containing residue mimetics can also be generated by reaction with imidoesters, such as methyl picolinimide, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transamidase-catalyzed reactions with glyoxylate. Mimetics of methionine can be generated by reaction with, e.g., methionine sulfoxide. Mimetics of proline include, e.g., pipecolic acid, thiazolidine carboxylic acid, 3- or 4- hydroxy proline, dehydroproline, 3- or 4-methylproline, or 3,3-dimethylproline. Histidine residue mimetics can be generated by reacting histidyl with, e.g., diethylprocarbonate or para-bromophenacyl bromide. Other mimetics include, e.g., those generated by hydroxylation of proline and lysine; phosphorylation of the hydroxyl groups of seryl or threonyl residues; methylation of the alpha-amino groups of lysine, arginine and histidine; acetylation of the N-terminal amine;

methylation of main chain amide residues or substitution with N-methyl amino acids; or amidation of C-terminal carboxyl groups.

A component of a polypeptide of the invention can also be replaced by an amino acid (or peptidomimetic residue) of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (which can also be referred to as the R or S, depending upon the structure of the chemical entity) can be replaced with the amino acid of the same chemical structural type or a peptidomimetic, but of the opposite chirality, referred to as the D- amino acid, but which can additionally be referred to as the R- or S- form.

The skilled artisan will recognize that individual synthetic residues and polypeptides incorporating these mimetics can be synthesized using a variety of procedures and methodologies, which are well described in the scientific and patent literature, *e.g.*, Organic Syntheses Collective Volumes, Gilman, et al. (Eds) John Wiley & Sons, Inc., NY. Peptides and peptide mimetics of the invention can also be synthesized using combinatorial methodologies. Various techniques for generation of peptide and peptidomimetic libraries are well known, and include, *e.g.*, multipin, tea bag, and split-couple-mix techniques; see, *e.g.*, al-Obeidi (1998) Mol. Biotechnol. 9:205-223; Hruby (1997) Curr. Opin. Chem. Biol. 1:114-119; Ostergaard (1997) Mol. Divers. 3:17-27; Ostresh (1996) Methods Enzymol. 267:220-234. Modified peptides of the invention can be further produced by chemical modification methods, see, *e.g.*, Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med. 19:373-380; Blommers (1994) Biochemistry 33:7886-7896.

Peptides and polypeptides of the invention can also be synthesized and expressed as fusion proteins with one or more additional domains linked thereto for, *e.g.*, producing a more immunogenic peptide, to more readily isolate a recombinantly synthesized peptide, to identify and isolate antibodies and antibody-expressing B cells, and the like. Detection and purification facilitating domains include, *e.g.*, metal chelating peptides such as polyhistidine tracts and histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequences such as Factor Xa or enterokinase (Invitrogen, San Diego CA) between a purification domain and the motif-comprising peptide or polypeptide to facilitate purification. For example, an expression vector can include an

epitope-encoding nucleic acid sequence linked to six histidine residues followed by a thioredoxin and an enterokinase cleavage site (see *e.g.*, Williams (1995) Biochemistry 34:1787-1797; Dobeli (1998) Protein Expr. Purif. 12:404-14). The histidine residues facilitate detection and purification while the enterokinase cleavage site provides a means for purifying the epitope from the remainder of the fusion protein. Technology pertaining to vectors encoding fusion proteins and application of fusion proteins are well described in the scientific and patent literature, see *e.g.*, Kroll (1993) DNA Cell. Biol., 12:441-53.

The invention provides methods for inhibiting the activity of a Chk1 kinase or a Chk2 kinase. The invention also provides methods for screening for compositions that inhibit the activity of, or bind to (*e.g.*, bind to the active site), Chk1 kinase and/or a Chk2 kinase. The amino acid sequence of human Chk1 kinase is

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MAVPFVEDWDLVQTLGEGAYGEVQLAVNRVTEEA VAVKIVDMKR
AVDCPENIKKEICINKMLNHENVVKFYGHRREGNIQYLFLEYCSGGELFDRIEPDIGM
PEPDAQRRFFHQLMAGVVYLHGIGITHRDIKPENLLLDERDNLKISDFGLATVFRYNNR
ERLLNKMCGTLPYVAPELLKRREFHAEPVDVWSCGIVLTAMLAGELPWDQPSDSCQEY
SDWKEKKTYLNPWKKIDSAPLALLHKILVENPSARITIPDIKKDRWYNKPLKKGAKRP
RVTSGGVSESPSGFSKHIQSNLDFSPVNSASSEENVKYSSSQPEPRTGLSLWDTSPSY
IDKL VQG ISFSQPTCPDHMLLNSQLLGTGSSQN PWQRLVKRMTRFFT KL DADKSYQC
LKETCEKLG YQWKSCMNQVTISTTDRRNNKLIFKVN LLEMDDKILVDFRLSKGDGLE
FKRHFLKIKGKLIDIVSSQKVWLPAT (SEQ ID NO:3)
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See also, Sanchez (1997) Science 277:1497-1501; Genbank Accession Nos. AF 016582; AAC 51736; NP 001265, NM 001274.

The amino acid sequence of human Chk2 kinase is

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MSRES DVEAQQSHGSSACSQPHG SVTQSQGSSSSQSQGISSSSTS
MPNSSQSSHSSSGTLSSLETVSTQELYSIPEDQEPEDEPEEPTPAPWARLWALQDG
FANLECVNDNYWFG RDKSCEYCFDEPLLKRTDKYRTYSKKHFRIFREVGPKN SYIAYI
EDHSGNGTFVNTELVGKGKRRPLNNNSEIALSLSRNKVFVFFDLTVDDQSVYPKALRD
EYIMSKTLGSGACGEVKLA FERKTCKKVAIKIISKRFKAIGSAREADPALNVETEIEI
LKKLNHP CIIKKNFFDAEDYYIVLELMEGGELFDKVVG NKRLKEATCKLYFYQMLLA
VQYLHENGIIHRDLKPENVLLSSQEEDCLIKITDFGHSKILGETSLMRTL CGTPTYLA
PEVLVSVGTAGYNRAVDCWSLGVILFICLSGYPPFSEHRTQVSLKDQITSGKYNFIPE
VWAEVSEKALDLVKLLVDPKARFTTEEALRHPWLQDEDMKRKFQDLLSEENESTAL
PQVLAQPSTSRKRPREGEAEGAETTKRPAVCAAVL (SEQ ID NO:4)
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See also Brown (1999) Proc. Natl. Acad. Sci. USA 96:3745-3750; Chaturvedi (1999) Oncogene 18:4047-4054; Genbank Accession Nos. NP 009125; NM 007194.

Antibody Generation

The invention provides antibodies that specifically bind to the peptides and polypeptides of the invention. These antibodies can be used to identify the presence of these peptides and polypeptides. The peptides and polypeptides of the invention can be used as immunogens to generate antibodies specific for a corresponding Cdc25C phosphatase. The anti-peptide antibodies of the invention can be used to generate anti-idiotypic antibodies that specifically bind to active sites of Chk1 or Chk2 kinase.

Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art and described in the scientific and patent literature, see, e.g., Coligan, CURRENT PROTOCOLS IN IMMUNOLOGY, Wiley/Greene, NY (1991); Stites (eds.) BASIC AND CLINICAL IMMUNOLOGY (7th ed.) Lange Medical Publications, Los Altos, CA ("Stites"); Goding, MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE (2d ed.) Academic Press, New York, NY (1986); Kohler (1975) Nature 256:495; Harlow (1988) ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publications, New York. Antibodies can be generated *in vitro*, e.g., using recombinant antibody binding site expressing phage display libraries, in addition to the traditional *in vivo* methods using animals. See, e.g., Huse (1989) Science 246:1275; Ward (1989) Nature 341:544; Hoogenboom (1997) Trends Biotechnol. 15:62-70; Katz (1997) Annu. Rev. Biophys. Biomol. Struct. 26:27-45. Human antibodies can be generated in mice engineered to produce only human antibodies, as described by, e.g., U.S. Patent No. 5,877,397; 5,874,299; 5,789,650; and 5,939,598. B-cells from these mice can be immortalized using standard techniques (e.g., by fusing with an immortalizing cell line such as a myeloma or by manipulating such B-cells by other techniques to perpetuate a cell line) to produce a monoclonal human antibody-producing cell. See, e.g., U.S. Patent No. 5,916,771; 5,985,615. For making chimeric, e.g., "humanized," antibodies, see e.g., U.S. Patent Nos. 5,811,522; 5,789,554; 5,861,155. Alternatively, recombinant antibodies can also be expressed by transient or stable expression vectors in mammalian, including human, cells as in Norderhaug (1997) J. Immunol. Methods 204:77-87; Boder (1997) Nat. Biotechnol. 15:553-557; see also U.S. Patent No. 5,976,833

Screening for candidate compounds

The invention provides compositions and methods for screening for potential therapeutic compounds ("candidate compounds") to inhibit or abrogate Chk1 and/or Chk2/Cds1 kinase activity and/or the G2 cell cycle arrest checkpoint. For example, the screening can involve *in vitro* or *in vivo* assays wherein Chk1 and Chk2/Cds1 kinases phosphorylate peptides and polypeptides comprising the motifs of the invention; see Example 1, below. Inhibitors of peptide phosphorylation are candidate compounds. Alternatively, assays incorporating the experiments, or variations thereof, as set forth in Example 1, below, can be designed to assay for candidate compounds which can inhibit or abrogate Chk1 and/or Chk2/Cds1 kinase activity and/or the G2 cell cycle arrest checkpoint.

In one embodiment, the peptides and polypeptides of the invention can be bound to a solid support. Solid supports can include, e.g., membranes (e.g., nitrocellulose or nylon), a microtiter dish (e.g., PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dip stick (e.g., glass, PVC, polypropylene, polystyrene, latex and the like), a microfuge tube, or a glass, silica, plastic, metallic or polymer bead or other substrate such as paper. One solid support uses a metal (e.g., cobalt or nickel)-comprising column which binds with specificity to a histidine tag engineered onto a peptide.

Adhesion of peptides to a solid support can be direct (i.e. the protein contacts the solid support) or indirect (a particular compound or compounds are bound to the support and the target protein binds to this compound rather than the solid support). Peptides can be immobilized either covalently (e.g., utilizing single reactive thiol groups of cysteine residues (see, e.g., Colliuod (1993) Bioconjugate Chem. 4:528-536) or non-covalently but specifically (e.g., via immobilized antibodies (see, e.g., Schuhmann (1991) Adv. Mater. 3:388-391; Lu (1995) Anal. Chem. 67:83-87; the biotin/streptavidin system (see, e.g., Iwane (1997) Biophys. Biochem. Res. Comm. 230:76-80); metal chelating, e.g., Langmuir-Blodgett films (see, e.g., Ng (1995) Langmuir 11:4048-55); metal-chelating self-assembled monolayers (see, e.g., Sigal (1996) Anal. Chem. 68:490-497) for binding of polyhistidine fusions.

Indirect binding can be achieved using a variety of linkers which are commercially available. The reactive ends can be any of a variety of functionalities including, but not limited to: amino reacting ends such as N-hydroxysuccinimide (NHS) active esters, imidoesters, aldehydes, epoxides, sulfonyl halides, isocyanate, isothiocyanate,

and nitroaryl halides; and thiol reacting ends such as pyridyl disulfides, maleimides, thiophthalimides, and active halogens. The heterobifunctional crosslinking reagents have two different reactive ends, e.g., an amino-reactive end and a thiol-reactive end, while homobifunctional reagents have two similar reactive ends, e.g., bismaleimido-hexane (BMH) which permits the cross-linking of sulfhydryl-containing compounds. The spacer can be of varying length and be aliphatic or aromatic. Examples of commercially available homobifunctional cross-linking reagents include, but are not limited to, the imidoesters such as dimethyl adipimidate dihydrochloride (DMA); dimethyl pimelimidate dihydrochloride (DMP); and dimethyl suberimidate dihydrochloride (DMS). Heterobifunctional reagents include commercially available active halogen-NHS active esters coupling agents such as N-succinimidyl bromoacetate and N-succinimidyl (4-iodoacetyl)aminobenzoate (SIAB) and the sulfosuccinimidyl derivatives such as sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB) (Pierce). Another group of coupling agents is the heterobifunctional and thiol cleavable agents such as N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Pierce Chemicals, Rockford, IL).

Antibodies can be used for binding polypeptides and peptides of the invention to a solid support. This can be done directly by binding peptide-specific antibodies to the column or it can be done by creating fusion protein chimeras comprising motif-containing peptides linked to, e.g., a known epitope (e.g., a tag (e.g., FLAG, myc) or an appropriate immunoglobulin constant domain sequence (an "immunoadhesin," see, e.g., Capon (1989) *Nature* 377:525-531 (1989)).

There are a variety of assay formats that can be used to screen for "candidate compounds" to inhibit or abrogate Chk1 and/or Chk2/Cds1 kinase activity and/or the G2 cell cycle arrest checkpoint.. For example, as discussed above, compounds that inhibit the phosphorylation of the motif-comprising peptides of the invention can be candidate compounds. Alternatively, compounds that specifically bind to the motifs of the invention can be candidate compounds. For a general description of different formats for binding assays, see, e.g., BASIC AND CLINICAL IMMUNOLOGY, 7th Ed. (D. Stiles and A. Terr, ed.)(1991); ENZYME IMMUNOASSAY, E.T. Maggio, ed., CRC Press, Boca Raton, Florida (1980); and "Practice and Theory of Enzyme Immunoassays" in P. Tijssen, LABORATORY

TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY, Elsevier Science Publishers, B.V. Amsterdam (1985).

Combinatorial chemical libraries

Combinatorial chemical libraries are one means to assist in the generation of new chemical compound leads, i.e., compounds that inhibit Chk1 and/or Chk2/Cds1 kinase and/or inhibit or abrogate the G2 cell cycle arrest checkpoint. A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks called amino acids in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks. For example, the systematic, combinatorial mixing of 100 interchangeable chemical building blocks results in the theoretical synthesis of 100 million tetrameric compounds or 10 billion pentameric compounds (see, e.g., Gallop et al. (1994) 37(9): 1233-1250). Preparation and screening of combinatorial chemical libraries are well known to those of skill in the art, see, e.g., U.S. Patent No. 6,004,617; 5,985,356. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (see, e.g., U.S. Patent No. 5,010,175; Furka (1991) Int. J. Pept. Prot. Res., 37: 487-493, Houghton et al. (1991) Nature, 354: 84-88). Other chemistries for generating chemical diversity libraries include, but are not limited to: peptoids (see, e.g., WO 91/19735), encoded peptides (see, e.g., WO 93/20242), random bio-oligomers (see, e.g., WO 92/00091), benzodiazepines (see, e.g., U.S. Patent No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (see, e.g., Hobbs (1993) Proc. Nat. Acad. Sci. USA 90: 6909-6913), vinylogous polypeptides (see, e.g., Hagihara (1992) J. Amer. Chem. Soc. 114: 6568), non-peptidal peptidomimetics with a Beta- D- Glucose scaffolding (see, e.g., Hirschmann (1992) J. Amer. Chem. Soc. 114: 9217-9218), analogous organic syntheses of small compound libraries (see, e.g., Chen (1994) J. Amer. Chem. Soc. 116: 2661), oligocarbamates (see, e.g., Cho (1993) Science 261:1303), and/or peptidyl phosphonates (see, e.g., Campbell (1994) J. Org. Chem. 59: 658). See also Gordon (1994) J. Med. Chem. 37:1385; for nucleic acid libraries, peptide nucleic acid libraries, see, e.g., U.S. Patent No. 5,539,083; for antibody libraries, see, e.g.,

Vaughn (1996) Nature Biotechnology 14:309-314; for carbohydrate libraries, see, e.g., Liang et al. (1996) Science 274: 1520-1522, U.S. Patent No. 5,593,853; for small organic molecule libraries, see, e.g., for isoprenoids U.S. Patent 5,569,588; for thiazolidinones and metathiazanones, U.S. Patent No. 5,549,974; for pyrrolidines, U.S. Patent Nos. 5,525,735 and 5,519,134; for morpholino compounds, U.S. Patent No. 5,506,337; for benzodiazepines U.S. Patent No. 5,288,514.

Devices for the preparation of combinatorial libraries are commercially available (see, e.g., U.S. Patent No. 6,045,755; 5,792,431 ; 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). A number of robotic systems have also been developed for solution phase chemistries. These systems include automated workstations, e.g., like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Hewlett-Packard, Palo Alto, Calif.) which mimic the manual synthetic operations performed by a chemist. Any of the above devices are suitable for use with the present invention. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art. In addition, numerous combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

Formulation and Administration of Pharmaceutical Compositions

In one embodiment, the peptides and polypeptides of the invention are combined with a pharmaceutically acceptable carrier (excipient) to form a pharmacological composition. Pharmaceutically acceptable carriers can contain a physiologically acceptable compound that acts to, e.g., stabilize, or increase or decrease the absorption or clearance rates of the pharmaceutical compositions of the invention. Physiologically acceptable compounds can include, e.g., carbohydrates, such as glucose, sucrose, or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins, compositions that reduce the clearance or hydrolysis of the peptides or polypeptides, or excipients or other stabilizers and/or buffers. Detergents can also be used to stabilize or to increase or decrease the absorption of the pharmaceutical composition, including liposomal carriers. Pharmaceutically acceptable carriers and formulations for peptides and polypeptide are known to the skilled artisan and are described in detail in the scientific and patent literature, see e.g., the latest edition of Remington's Pharmaceutical Science, Mack Publishing Company, Easton, Pennsylvania ("Remington's").

Other physiologically acceptable compounds include wetting agents, emulsifying agents, dispersing agents or preservatives which are particularly useful for preventing the growth or action of microorganisms. Various preservatives are well known and include, e.g., phenol and ascorbic acid. One skilled in the art would appreciate that the choice of a pharmaceutically acceptable carrier including a physiologically acceptable compound depends, for example, on the route of administration of the peptide or polypeptide of the invention and on its particular physio-chemical characteristics.

In one embodiment, a solution of peptide or polypeptide of the invention is dissolved in a pharmaceutically acceptable carrier, e.g., an aqueous carrier if the composition is water-soluble. Examples of aqueous solutions that can be used in formulations for enteral, parenteral or transmucosal drug delivery include, e.g., water, saline, phosphate buffered saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions and the like. The formulations can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents, detergents and the like. Additives can also include additional active ingredients such as bactericidal agents, or stabilizers. For example, the solution can contain

sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate or triethanolamine oleate. These compositions can be sterilized by conventional, well-known sterilization techniques, or can be sterile filtered. The resulting aqueous solutions can be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The concentration of peptide in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

Solid formulations can be used for enteral (oral) administration. They can be formulated as, e.g., pills, tablets, powders or capsules. For solid compositions, conventional nontoxic solid carriers can be used which include, e.g., pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10% to 95% of active ingredient (e.g., peptide). A non-solid formulation can also be used for enteral administration. The carrier can be selected from various oils including those of petroleum, animal, vegetable or synthetic origin, e.g., peanut oil, soybean oil, mineral oil, sesame oil, and the like. Suitable pharmaceutical excipients include e.g., starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol.

Peptides and polypeptides of the invention, when administered orally, can be protected from digestion. This can be accomplished either by complexing the peptide or polypeptide with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the peptide or complex in an appropriately resistant carrier such as a liposome. Means of protecting compounds from digestion are well known in the art, see, e.g., Fix (1996) *Pharm Res.* 13:1760-1764; Samanen (1996) *J. Pharm. Pharmacol.* 48:119-135; U.S. Patent 5,391,377, describing lipid compositions for oral delivery of therapeutic agents (liposomal delivery is discussed in further detail, *infra*).

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be

permeated can be used in the formulation. Such penetrants are generally known in the art, and include, e.g., for transmucosal administration, bile salts and fusidic acid derivatives. In addition, detergents can be used to facilitate permeation. Transmucosal administration can be through nasal sprays or using suppositories. See, e.g., Sayani (1996) "Systemic delivery of peptides and proteins across absorptive mucosae" Crit. Rev. Ther. Drug Carrier Syst. 13:85-184. For topical, transdermal administration, the agents are formulated into ointments, creams, salves, powders and gels. Transdermal delivery systems can also include, e.g., patches.

The peptides and polypeptide complexes can also be administered in sustained delivery or sustained release mechanisms, which can deliver the formulation internally. For example, biodegradable microspheres or capsules or other biodegradable polymer configurations capable of sustained delivery of a peptide can be included in the formulations of the invention (see, e.g., Putney (1998) Nat. Biotechnol. 16:153-157).

For inhalation, the peptide or polypeptide can be delivered using any system known in the art, including dry powder aerosols, liquids delivery systems, air jet nebulizers, propellant systems, and the like. See, e.g., Patton (1998) Biotechniques 16:141-143; product and inhalation delivery systems for polypeptide macromolecules by, e.g., Dura Pharmaceuticals (San Diego, CA), Aradigm (Hayward, CA), Aerogen (Santa Clara, CA), Inhale Therapeutic Systems (San Carlos, CA), and the like. For example, the pharmaceutical formulation can be administered in the form of an aerosol or mist. For aerosol administration, the formulation can be supplied in finely divided form along with a surfactant and propellant. In another embodiment, the device for delivering the formulation to respiratory tissue is an inhaler in which the formulation vaporizes. Other liquid delivery systems include, e.g., air jet nebulizers.

In preparing pharmaceuticals of the present invention, a variety of formulation modifications can be used and manipulated to alter pharmacokinetics and biodistribution. A number of methods for altering pharmacokinetics and biodistribution are known to one of ordinary skill in the art. Examples of such methods include protection of the complexes in vesicles composed of substances such as proteins, lipids (for example, liposomes, see below), carbohydrates, or synthetic polymers (discussed above). For a general discussion of pharmacokinetics, see, e.g., Remington's, Chapters 37-39.

The peptide and polypeptide complexes used in the methods of the invention can be delivered alone or as pharmaceutical compositions by any means known in the art, e.g., systemically, regionally, or locally (e.g., directly into, or directed to, a tumor); by intraarterial, intrathecal (IT), intravenous (IV), parenteral, intra-pleural cavity, topical, oral, or local administration, as subcutaneous, intra-tracheal (e.g., by aerosol) or transmucosal (e.g., buccal, bladder, vaginal, uterine, rectal, nasal mucosa). Actual methods for preparing administrable compositions will be known or apparent to those skilled in the art and are described in detail in the scientific and patent literature, see e.g., Remington's. For a "regional effect," e.g., to focus on a specific organ, one mode of administration includes intra-arterial or intrathecal (IT) injections, e.g., to focus on a specific organ, e.g., brain and CNS (see e.g., Gurun (1997) *Anesth Analg.* 85:317-323). For example, intra-carotid artery injection is preferred where it is desired to deliver a peptide or polypeptide complex of the invention directly to the brain. Parenteral administration is a preferred route of delivery if a high systemic dosage is needed. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in detail, in e.g., Remington's. See also, Bai (1997) *J. Neuroimmunol.* 80:65-75; Warren (1997) *J. Neurol. Sci.* 152:31-38; Tonegawa (1997) *J. Exp. Med.* 186:507-515.

In one embodiment, the pharmaceutical formulations comprising peptides or polypeptides of the invention are incorporated in lipid monolayers or bilayers, e.g., liposomes, see, e.g., U.S. Patent No. 6,110,490; 6,096,716; 5,283,185; 5,279,833. The invention also provides formulations in which water soluble peptides or complexes have been attached to the surface of the monolayer or bilayer. For example, peptides can be attached to hydrazide- PEG- (distearoylphosphatidyl) ethanolamine- containing liposomes (see, e.g., Zalipsky (1995) *Bioconjug. Chem.* 6:705-708). Liposomes or any form of lipid membrane, such as planar lipid membranes or the cell membrane of an intact cell, e.g., a red blood cell, can be used. Liposomal formulations can be by any means, including administration intravenously, transdermally (see, e.g., Vutla (1996) *J. Pharm. Sci.* 85:5-8), transmucosally, or orally. The invention also provides pharmaceutical preparations in which the peptides and/or complexes of the invention are incorporated within micelles and/or liposomes (see, e.g., Suntres (1994) *J. Pharm. Pharmacol.* 46:23-28; Woodle (1992) *Pharm. Res.* 9:260-265). Liposomes and liposomal formulations can be prepared according to standard methods and

are also well known in the art, see, e.g., Remington's; Akimaru (1995) Cytokines Mol. Ther. 1:197-210; Alving (1995) Immunol. Rev. 145:5-31; Szoka (1980) Ann. Rev. Biophys. Bioeng. 9:467, U.S. Pat. Nos. 4, 235,871, 4,501,728 and 4,837,028.

Treatment Regimens: Pharmacokinetics

5 The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. Dosages for typical peptide and polypeptide pharmaceutical compositions are well known to those of skill in the art. Such dosages are typically advisory in nature and are adjusted depending on the particular therapeutic context, patient tolerance, etc. The amount of peptide or polypeptide adequate to
10 accomplish this is defined as a "therapeutically effective dose." The dosage schedule and amounts effective for this use, i.e., the "dosing regimen," will depend upon a variety of factors, including the stage of the disease or condition, the severity of the disease or condition, the general state of the patient's health, the patient's physical status, age, pharmaceutical formulation and concentration of active agent, and the like. In calculating the
15 dosage regimen for a patient, the mode of administration also is taken into consideration. The dosage regimen must also take into consideration the pharmacokinetics, i.e., the pharmaceutical composition's rate of absorption, bioavailability, metabolism, clearance, and the like. See, e.g., the latest Remington's; Egleton (1997) "Bioavailability and transport of peptides and peptide drugs into the brain" Peptides 18:1431-1439; Langer (1990) Science
20 249:1527-1533.

 In therapeutic applications, compositions are administered to a patient suffering from a cancer in an amount sufficient to at least partially arrest the disease and/or its complications. For example, in one embodiment, a soluble peptide pharmaceutical composition dosage for intravenous (IV) administration would be about 0.01 mg/hr to about
25 1.0 mg/hr administered over several hours (typically 1, 3, or 6 hours), which can be repeated for weeks with intermittent cycles. Considerably higher dosages (e.g., ranging up to about 10 mg/ml) can be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ, e.g., the cerebrospinal fluid (CSF).

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1: Administration of peptides of the invention to selectively sensitize cancer cells to DNA damaging agents

The invention provides compositions and methods for sensitizing cells, particularly cells with an impaired G1 cell cycle arrest checkpoint, such as cancer cells, to DNA damaging agents. The following example describes studies which demonstrate that the compositions and methods of the invention are effective for selectively killing cancer cells (versus normal cells, which have an unimpaired G1 checkpoint). Specifically, these experiments describes the synthesis and use of two exemplary polypeptides of the invention. Two peptides corresponding to amino acids 211 to 221 of human Cdc25C (SEQ ID NO:1) fused with a part of HIV-1-TAT (SEQ ID NO:5). These peptides were demonstrated to inhibit hChk1 kinase (SEQ ID NO:3) and Chk2/Hu-Cds1 (SEQ ID NO:4) kinase activity *in vitro* and to specifically abrogate the G2 checkpoint *in vivo*.

Chemicals and reagents. Bleomycin and colchicine were purchased from Wako Pure Chemical Co. (Osaka, Japan). Hydroxyurea was purchased from Sigma Chemical Co. (St. Louis, MO). These chemicals were dissolved in distilled H₂O to 10, 5 and 50 mg/ml, respectively, and stored at 4°C. Antibodies against 14-3-3 β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Life Sciences (Arlington Heights, IL). Antibodies against HA and c-myc, and protein G-Sepharose were purchased from Santa Cruz Biotechnology and Amersham Pharmacia Biotech (Uppsala, Sweden), respectively.

Cell culture and plasmids. A human T-cell leukemia-derived cell line, Jurkat, was cultured in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (IBL: Immuno-Biological Laboratories, Gunma, Japan) at 37°C/5% CO₂. Human pancreatic epitheloid carcinoma-derived cell lines, MIA PaCa2 and PANC1, were cultured in Eagle's MEM (IWAKI, Chiba, Japan) and Dulbecco's modified Eagle's medium with 4 mM l-glucose (Sigma) and 1.0 mM sodium pyruvate (Life Technologies, Inc., Grand Island, NY), respectively, and supplemented with 10% fetal calf serum at 37°C/5% CO₂. Normal human

peripheral blood lymphocytes were collected by Ficoll-Paque (Amersham Pharmacia Biotech) density gradient. Two million cells/ml were cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37°C/5% CO₂ in the presence of 5 µg/ml PHA (Life Technologies, Inc.) for a week. Baculovirus lysates that include HA-tagged hChk1 (SEQ ID NO:3) or c-myc-tagged Chk2/HuCds1 (SEQ ID NO:4) and plasmid for GST-Cdc25C (amino acid 200-256) were made as described in Matsuoka (1998) Science 282:1893-1897, and provided by Dr. Makoto Nakanishi (Department of Biochemistry, Nagoya City University).

Peptides. TAT-S216 peptide was synthesized so that it contained an NH₂-terminal 11 amino acid TAT protein transduction domain (YGRKKRRQRRR (SEQ ID NO:5); see, e.g., Nagahara (1998) Nature Med. 4:1449-1452) followed by a corresponding amino acid 211 to 221 derived from the human Cdc25C amino acid sequence (SEQ ID NO:1) (S216; LYRSPASMPENL). Serine-216 residue was changed to alanine in TAT-S216A (S216A; LYRSPSMPENL) (SEQ ID NO:6). The Cdc25C portion was partially deleted and substituted with glycine in TAT-Control (GGRSPAMPE) (SEQ ID NO:7). All peptides were synthesized by Sawady Technology Co. (Tokyo, Japan).

Purification of recombinant GST-Cdc25C proteins. *Escherichia coli* DH5α cells were transformed by GST-Cdc25C (200-256) plasmid. The cells were incubated with 0.1 mM isopropyl β-D-thiogalactoside for 2 hr, harvested, and lysed with a buffer containing 50 mM Tris HCl (pH8.0), 100 mM NaCl, 0.5% NP-40, 5 µg/ml aprotinin, 5 µg/ml pepstatin A and 5 µg/ml leupeptin. The lysate was sonicated, centrifuged for clarification and incubated with glutathione-Sepharose 4B™ beads for 1 hr at 4°C and washed five times.

Kinase assay. HA-tagged hChk1 (SEQ ID NO:3) and c-myc-tagged Chk2/HuCds1 (SEQ ID NO:4) expressed in insect cells using recombinant baculovirus (see, e.g., Kaneko (1999) Oncogene 18:3673-3681) were purified by immunoprecipitation using anti-HA or anti-c-myc antibodies and protein G-Sepharose. Immune complex kinase reaction was done in PBS with 1 mM DTT, 1 mM MgCl₂ and 100 µCi of [γ -³²P] ATP (Amersham; 6000Ci/mmol) plus purified 1 µM GST-Cdc25C or 10 µM Cdc25C peptide (amino acid 211 to 221 of Cdc25C (SEQ ID NO:1); LYRSPSMPENL, Sawady Technology Co.) substrates at 30°C for 15 min in the presence of 10 µM TAT-S216, TAT-S216A or TAT-Control. After the reaction, samples were separated in 12% or 15% SDS-PAGE and autoradiographed to detect GST-Cdc25C or peptide phosphorylation.

Cell-cycle analysis. The cell cycle status of the cells treated with peptides and/or bleomycin or colchicine was analyzed by FACS, as described by Kawabe (1997) Nature 385:454-458. In brief, two million Jurkat cells were re-suspended and incubated in 300 μ l Krishan's solution (0.1% Sodium citrate, 50 μ g/ml PI, 20 μ g/ml RNase A and 0.5% NP-40; see supra) for 1 hr at 4°C and analyzed by FACScan™ (Beckton Dickinson, Mountain View, CA) with the program CELLQuest™ (Beckton Dickinson).

Histone H1 kinase assay. Ten million Jurkat cells were treated with hydroxyurea (100 μ g/ml), bleomycin (10 μ g/ml), or colchicine (5 μ g/ml) with or without addition of TAT-S216A, TAT-S216 or TAT-Control (10 μ M) for 6 hr. The cells were washed in cold PBS and lysed at 4°C in 1 ml of buffer A (50 mM Tris pH 8, 2 mM DTT, 5 mM EDTA, 100 mM NaCl, 0.5% NP40, 20 mM Na₃V0₄, 50 mM NaF, 4 μ M Okadaic acid, 5 μ g/ml aprotinin, 5 μ g/ml pepstatin A and 5 μ g/ml leupeptin.). Twenty microliter of p13^{suc1} agarose beads (Upstate Biotechnology., Saranac, NY) were added to the cleared lysates, incubated for 4 hr at 4°C, and washed five times with buffer A without 5 mM EDTA, 20 mM Na₃V0₄, 50 mM NaF, 4 μ M Okadaic acid. Histone H1 kinase activity on the beads were analyzed by using Cdc2 kinase assay kit (Upstate Biotechnology) with [γ -³²P] ATP followed by 12% SDS-PAGE electrophoresis, and autoradiographed to detect the phosphorylated Histone H1.

Cell cytotoxicity assay. MIA PaCa2 and PANC1 cells (3x10³/well) were plated in 96-well microtiter plates. After an overnight adherence, cells were treated with bleomycin (10 μ g/ml) with or without the indicated TAT-peptides at various time points up to 96 hr. Cytotoxicity and cell survival were determined by the 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay (Cell Proliferation Kit II™: Boehringer Mannheim, Germany), which was done according to company's protocol and Scudiero (1988) Cancer Res. 48:4827-4833.

TAT-S216 and TAT-S216A peptides inhibit hChk1 and Chk2/HuCds1 kinase activities

To inhibit hChk1 (SEQ ID NO:3) and Chk2/HuCds1 (SEQ ID NO:4) kinase activities and to abrogate DNA damage-induced-G2 arrest, synthetic peptides comprising amino acid residues 211 to 221 of Cdc25C (SEQ ID NO:1) and a variation of the TAT protein transduction domain (YGRKKRRQRRR (SEQ ID NO:5) (TAT-S216) were generated.

The results are shown in Figure 1: TAT-S216A and TAT-S216 peptides inhibit hChk1 and Chk2/HuCds1 kinase activities *in vitro*. Figure 1A, sequences of the peptides. Figure 1B, *in vitro* phosphorylation analysis using GST-Cdc25C and purified hChk1. GST-Cdc25C (amino acid 200-256) that was produced in *E. coli* (DH5 α) was used as substrate (1 μ M). Immune complex kinase reaction was done in the presence of TAT-S216A (10 μ M) or TAT-S216 (10 μ M). Figure 1C, *in vitro* phosphorylation analysis of hChk1 and Chk2/HuCds1 using synthesized Cdc25C peptide corresponding amino acid 211-221 of Cdc25C (LYRSPSPENL) as a substrate (10 μ M).

A TAT-S216A peptide (S216A; LYRSPSPENL, (SEQ ID NO:6)), in which serine residue 216 was substituted by alanine was devised to stabilize the transient status of its interaction with hChk1 (SEQ ID NO:3) and Chk2/HuCds1 (SEQ ID NO:4) (Fig. 1A). This TAT peptide was included to efficiently transduce these peptides into cells (see, e.g., Nagahara (1998) *supra*). This sequence is known to facilitate the uptake of heterologous proteins across the cell membrane. As a control peptide, part of the Cdc25C portion of this peptide was deleted (TAT-Control).

As shown in Fig. 1B, hChk1 (SEQ ID NO:3) was capable of phosphorylating a Cdc25C protein (residues 200-256) (SEQ ID NO:1) fused to GST. Serine-216 on Cdc25C (SEQ ID NO:1) is the major phosphorylation site of this fusion protein *in vivo* (see, e.g., Furnari (1997) *Science* 277:1495-1497; Sanchez (1997) *Science* 277:1497-1501; Peng (1997) *Science* 277:1501-1505).

In Fig. 1B, both TAT-S216 and TAT-S216A inhibited the phosphorylation of Cdc25C by baculovirus-produced hChk1 (SEQ ID NO:3). TAT-S216 but not TAT-S216A was efficiently phosphorylated by hChk1, suggesting that serine-216 on TAT-S216 was phosphorylated by hChk1 and TAT-S216 would competitively inhibit substrate

phosphorylation at excess molar ratio if present in great enough quantity. TAT-Control peptide did not inhibit hChk1 kinase activity.

As shown in Fig. 1C, TAT-S216A significantly inhibited phosphorylation of Cdc25C peptide (residues 200-256) (SEQ ID NO:1) mediated by hChk1 (SEQ ID NO:3) and Chk2/Hu-Cds1 (SEQ ID NO:4) even at a low stoichiometry (at four times more molar excess of TAT-S216A peptide against substrate Cdc25C peptide).

Abrogation of DNA damage-induced G2 checkpoint by TAT-S216 and TAT-S216A peptides

The cell cycle status of the cells treated with TAT-S216A or TAT-S216 upon the DNA damage-induced G2 arrest was analyzed by FACS analysis. Histone H1 kinase activities of these cells were simultaneously monitored. Jurkat cells arrested exclusively at G2 by bleomycin (10 µg/ml) treatment, because it does not have functional p53. Results are shown in Figure 2: abrogation of DNA damage-induced G2 arrest by TAT-S216A and TAT-S216 peptides. Figure 2A, FACS analysis of Jurkat cells treated with bleomycin and peptides. Cells were treated with bleomycin (10 µg/ml) with or without peptides (10 µM) for 20 hr. B, histone H1 kinase analysis. Cell lysates were prepared from the cells treated with the indicated reagent for 6 hr. Concentrations used were: hydroxyurea (HU), 100 µg/ml; bleomycin (Bleo), 10 µg/ml; colchicine, 5 µg/ml; TAT-S216A and TAT-S216, 10 µM. C, FACS analysis of colchicine -and peptide-treated cells. Jurkat cells were treated with colchicine (5 µg/ml) with or without peptide (10 µM) for 20 hr.

As shown in Fig. 2A, G2 arrest was completely abrogated by the addition of TAT-S216A or TAT-S216 in response to bleomycin. G2 arrest was abrogated at any time point between 12 and 48 hr by the treatment with TAT-S216A or TAT-S216. Jurkat cells treated with bleomycin together with TAT-Control arrested at G2 similarly to the cells treated with bleomycin alone.

We also observed that either TAT-S216A or TAT-S216 also abrogated G2 arrest induced by gamma-irradiation and cisplatin (gamma-irradiation, 5 Gy; cisplatin, 1 µg/ml for 1 hr treatment). To further analyze the effect of these peptides on G2/M transition, histone H1 kinase activity was monitored. Consistent with the above findings, although histone H1 kinase activity was decreased by the treatment with bleomycin or hydroxyurea, it was unchanged or rather increased by the treatment with bleomycin in the presence of TAT-

S216A or TAT-S216 (Fig. 2B). In the presence of TAT-Control peptide, the bleomycin treatment did not affect with H1 kinase activity.

As shown in Fig. 2C, The M-phase arrest of Jurkat cells induced by colchicine was not affected by the addition of TAT-S216 or TAT-S216A. These results demonstrate that TAT-S216A and TAT-S216 specifically abrogated the DNA damage-activated cell cycle G2 checkpoint by inhibiting hChk1 (SEQ ID NO:3) and/or Chk2/HuCds1 (SEQ ID NO:4) kinase activities.

Sensitization of Jurkat cells to the bleomycin-induced cell death by TAT-S216A and TAT-S216 peptides

The effect of TAT-S216A and TAT-S216 on the cell death induced by bleomycin was examined. The results are shown in Figure 3; Trypan blue dye exclusion analysis of Jurkat cells treated with bleomycin (A) or colchicine (B) with or without indicated peptides. Bars, SD Vertical axis, % viability of the cells; Bleo 5, bleomycin 5 μ g/ml; Bleo 10, bleomycin 10 μ g/ml; colchicine, 5 μ g/ml; TAT-S216 or TAT-S216A, 10 μ M of indicated peptide. Note that TAT-S216A and TAT-S216 peptides did not increase the cytotoxicity of bleomycin to normal cells. C, survival analysis of PHA blasts treated with bleomycin and peptides. Vertical axis, % viability of the cells determined by trypan blue dye exclusion analysis; horizontal axis, time in hours. Bleo 5, bleomycin 5 μ g/ml; Bleo 10, bleomycin 10 μ g/ml; TAT-S216 or TAT-S216A, 10 μ M of indicated peptide. D, FACS analysis of the cells treated with bleomycin and peptides. PHA-blasts were treated with bleomycin with or without peptides for 20 hr. Vertical axis, cell number; horizontal axis, DNA content indicated by propidium iodide staining.

As shown in Fig. 3A, the addition of TAT-S216A and TAT-S216 efficiently sensitized Jurkat cells to the bleomycin-induced cell death. Whereas bleomycin treatment at 5 or μ 10 g/ml killed Jurkat cells by only 27-30%, the addition of 10 μ M TAT-216A or TAT-S216 killed Jurkat cells by nearly 80%. In contrast, these peptide by themselves did not show any significant cytotoxicity. In addition, a control peptide TAT-Control did not affect the viability of bleomycin-treated Jurkat cells. Moreover, as expected from the result in Fig. 2C, either TAT-S216A or TAT-S216 did not affect the cytotoxicity by colchicine (Fig. 3B). This observation indicates that the cell death induced by these peptides in the presence of bleomycin was not attributable to a nonspecific cytotoxic effect.

TAT-S216 and TAT-S216A peptides did not affect the viability of normal cells

In order to confirm the specificity of the effect of these peptides on cancer cells in which the G1 checkpoint is abrogated, the effect of these peptides on normal human cells was investigated. Mitogen-activated normal human T lymphocytes (PHA blasts) were prepared by stimulating peripheral blood mononuclear cells obtained from a healthy donor with PHA for 1 week. These cells were treated with bleomycin (5 and 10 μ g/ml) in the presence or absence of either TAT-S216A or TAT-S216.

As shown in Fig. 3C, these peptides did not augment the cytotoxic effect of bleomycin, although these cells replicated as fast as Jurkat cells. As shown in Fig. 3D, PHA blasts treated with bleomycin (5 μ g/ml) arrested at G1 and S phase but not G2, presumably because of the activity of wild-type p53. When these cells were treated with TAT-S216 or TAT-S216A in addition to bleomycin, no further alteration of cell cycle pattern was observed.

Sensitization of pancreatic cancer cells to the bleomycin-induced cell death by TAT-S216A and TAT-S216 peptides

The effect of these peptides on two other p53-defective pancreatic cancer cell lines, MIA PaCa2 and PANC1 cells, was examined. Figure 4 shows the results of survival analysis of PANC1 (A) and MIA PaCa2 (B) cells treated with bleomycin and peptides. PANC1 and MIA PaCa2 cells were treated with bleomycin with or without the indicated peptide. The cell viability was determined by the 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate assay at the indicated times after addition of bleomycin and peptide. Bleo 60, bleomycin 60 μ g/ml; TAT-S216 or TAT-S216A, 10 μ M of indicated peptide. Bars, SD.

Although these pancreatic cancer cells are known to be resistant to various anti-cancer reagents, these cells could also be sensitized to the bleomycin-induced cell death by TAT-S216A and TAT-S216 (Fig. 4). Similarly, these peptides could sensitize these cells to the cell death induced by other DNA-damaging agents including cisplatin and gamma-irradiation.

In summary, these experiments demonstrated for the first time that short peptides that inhibit both hChk1 and Chk2/Hu-Cds1 kinase activities can specifically abrogate the DNA damage-induced G2 cell growth arrest checkpoint. These data also demonstrated

that the specific abrogation of the G2 checkpoint sensitized cancer cells to bleomycin, a DNA-damaging agent, without obvious effect on normal cell cycle and its viability. These observations indicate that these kinases involved in G2 cell cycle checkpoint are ideal targets for the specific abrogation of G2 checkpoint and that the peptides and polypeptides of the invention and their derivatives can be used in novel cancer therapy.

Example 2: Optimization of sequences for G2 abrogating peptides of the invention

The following example describes studies which identified exemplary G2 checkpoint-abrogating peptides of the invention. This was accomplished by using a computer analysis of the structure of human Chk2 kinase (SEQ ID NO:4) and the peptides of the invention.

The 3-dimensional structure of human Chk2 was predicted by comparing the primary and 3-D structure of another serine threonine kinase, PKA (PDB protein data base, Research Collaboratory for Structural Bioinformatics (RCSB), The National Science Foundation, Arlington, VA) (1CDK), using a computer program, MODELER™ (IMMD, Tokyo, Japan). The alignment of the peptides of the invention and hChk2 were predicted by comparing an alignment of hChk1 and various Cdc25C peptides as described by Chen (2000) "The 1.7 Å crystal structure of human cell cycle checkpoint kinase Chk1: implications for Chk1 regulation," Cell 100:681-92. By comparing the predicted structure of hChk2 with the peptides of the invention, it was predicted that there are four pockets on hChk2 that are important for the interaction with peptides, as shown in Figure 5, P1, P2, P3 and P4. The structure of these pockets was used to design and confirm the sequences of exemplary peptides of the invention

The ability of these peptides to abrogate the activity of Chk2 kinase, thereby imbuing the ability to abrogate the G2 cell cycle checkpoint, was demonstrated by their ability to act as a phosphorylation substrate for human Chk2 kinase. Exemplary peptides were directly synthesized (immobilized) on a membrane and contacted with human Chk2 kinase. Specifically, oligo-peptides with all sequences predicted by the 3-dimensional model were directly synthesized on a membrane by using an auto-spot-peptide-synthesizer, Model ASP-22 2 (ABiMED, Germany). The amount of peptide was about 0.1 micro-mol/cm².

The membrane was incubated with 2% Gly-Gly in PBS for 2 hours (hr) at room temperature (RT). Then, they were washed three times with 0.1% Tween-P BS™. The

“kination,” or “phosphorylation,” reaction was performed with a recombinant fusion protein Gst-Chk2 at a concentration of about 5 µg in 4 ml reaction buffer, 1 mM MgCl₂, 2% Gly-Gly and γ-³³P-ATP in PBS at RT for 1 hr. After the reaction, the membrane was washed 5 times with RIPA (1% SDS, 1% NP-40, 100 mM NaCl) and analyzed with a Bass 2500™ image analyzer (Fuji, Japan). The signal was graded to “-,” a “+,” a “++,” or a “+++.” Table 1 shows the peptide sequences that gave signals stronger than “++.” The peptides RYSLPPELSNM and LYRSPSAMPENL gave “+” signals by this analysis.

All of the following peptides were phosphorylated by human Chk2 kinase; in position “X” (corresponding to position X₈), wherein X = P, F, Y, or W, the signal was strongest (a “+++”) when X = the amino acid tyrosine (Y):

37-40 L Y R S P S H X E N L
 52-53 L Y S S P S Y X E N L
 92-95 L Y T S P S Y X E N L
 117-121 L Y T S P S H X E N L
 132-135 L Y H S P S Y X E N L
 1127-1130 W Y R S P S F X E N L
 1237-1240 W Y T S P S H X E N L
 372-375 L F T S P S Y X E N L
 637-640 F Y S S P S H X E N L
 642-645 F Y T S P S M X E N L
 648-651 F Y T S P S F X E N L
 652-655 F Y T S P S Y X E N L
 1202-1205 W Y T S P S M X E N L
 1207-1210 W Y T S P S F X E N L
 1212-1215 W Y T S P S Y X E N L

The best phosphorylation substrates were the peptides L Y R S P S Y Y E N L and W Y T S P S Y F E N L.

The following Table 1 is a complete list of tested peptides and results of the *in vitro* phosphorylation by human Chk2 kinase assay. Results are presented to the right of the peptide, below: a “+++” indicates the peptide was relatively highly phosphorylated; a “++” indicates the peptide was relatively less phosphorylated, a “+” indicates the peptide was

detectably significantly phosphorylated over negative control, and no indication indicates that a peptide was not significantly phosphorylated over negative control (note: the number immediately to the right of the peptide is the MW of the peptide).

Table 1

1 RYSLPPELSNM 1308.6	+	1 RYSLPPELSNM 1308.6
2 LYRSPSPMPENL 1308.6	+	2 LYRSPSPMPENL 1308.6
3 LYRSPSMFENL 1358.6	-	
4 LYRSPSMYENL 1374.6	-	
5 LYRSPSMWENL 1397.7	-	
7 LYRSPSFPENL 1324.5	-	
8 LYRSPSFFENL 1374.5	-	
9 LYRSPSFYENL 1390.5	-	
10 LYRSPSFWENL 1413.6	-	
12 LYRSPSYPENL 1340.5	+	
13 LYRSPSYFENL 1390.5	+	
14 LYRSPSYYENL 1406.5	+	
15 LYRSPSYWENL 1429.6	+	
17 LYRSPSDPENL 1292.4	-	
18 LYRSPSDFENL 1342.4	-	
19 LYRSPSDYENL 1358.4	-	
20 LYRSPSDWENL 1381.5	-	
22 LYRSPSEPENL 1306.4	-	
23 LYRSPSEFENL 1356.4	-	
24 LYRSPSEYENL 1372.4	-	
25 LYRSPSEWENL 1395.5	-	
27 LYRSPSNPENL 1291.5	+	
28 LYRSPSNEENL 1341.5	+	
29 LYRSPSNYENL 1357.5	+	
30 LYRSPSNWENL 1380.6	+	
32 LYRSPSQPENL 1305.5	-	
33 LYRSPSQFENL 1355.5	-	
34 LYRSPSQYENL 1371.5	-	
35 LYRSPSQWENL 1394.6	-	
37 LYRSPSHPENL 1314.5	+	

83	LYTSPSMFENL	1303.5
84	LYTSPSMYENL	1319.5
85	LYTSPSMWENL	1342.6
87	LYTSPSFPENL	1269.4
88	LYTSPSFFENL	1319.4
89	LYTSPSFYENL	1335.4
90	LYTSPSFWENL	1358.5
92	LYTSPSYPENL	1285.4
93	LYTSPSYFENL	1335.4
94	LYTSPSYYENL	1351.4
95	LYTSPSYWENL	1374.5
97	LYTSPSDPENL	1237.3
98	LYTSPSDFENL	1287.3
99	LYTSPSDYENL	1303.3
100	LYTSPSDWENL	1326.4
102	LYTSPSEPENL	1251.3
103	LYTSPSEFENL	1301.3
104	LYTSPSEYENL	1317.3
105	LYTSPSEWENL	1340.4
107	LYTSPSNPENL	1236.4
108	LYTSPSNFENL	1286.4
109	LYTSPSNYENL	1302.4
110	LYTSPSNWENL	1325.5
112	LYTSPSQPENL	1250.4
113	LYTSPSQFENL	1300.4
114	LYTSPSQYENL	1316.4
115	LYTSPSQWENL	1339.5
117	LYTSPSHPENL	1259.4
118	LYTSPSHFENL	1309.4
119	LYTSPSHYENL	1325.4
120	LYTSPSHWENL	1348.5
122	LYHSPSMPENL	1289.6
123	LYHSPSMFENL	1339.6
124	LYHSPSMYENL	1355.6
125	LYHSPSMWENL	1378.7
127	LYHSPSFPENL	1305.5

++	1240	WYTSPSHWENL	1421.6
++	372	LFTSPSYPENL	1269.4
++	373	LFTSPSYFENL	1319.4
++	374	LFTSPSYYENL	1335.4
++	375	LFTSPSYWENL	1358.5
++	637	FYSSPSHPENL	1280.2
++	638	FYSSPSHFENL	1330.2
++	639	FYSSPSHYENL	1346.2
++	640	FYSSPSHWENL	1369.3
+++	642	FYTSPSMPENL	1287.5
++	643	FYTSPSMFENL	1337.5
-	644	FYTSPSMYENL	1353.5
-	645	FYTSPSMWENL	1376.6
-	647	FYTSPSFPENL	1303.4
-	648	FYTSPSFFENL	1353.4
-	649	FYTSPSFYENL	1369.4
-	650	FYTSPSFWENL	1392.5
-	652	FYTSPSYPENL	1319.4
-	653	FYTSPSYFENL	1369.4
+	654	FYTSPSYYENL	1385.4
+	655	FYTSPSYWENL	1408.5
+	1202	WYTSPSMPENL	1326.6
+	1203	WYTSPSMFENL	1376.6
-	1204	WYTSPSMYENL	1392.6
-	1205	WYTSPSMWENL	1415.7
-	1207	WYTSPSFPENL	1342.5
-	1208	WYTSPSFFENL	1392.5
+	1209	WYTSPSFYENL	1408.5
+	1210	WYTSPSFWENL	1431.6
+	1212	WYTSPSYPENL	1358.5
+	1213	WYTSPSYFENL	1408.5
-	1214	WYTSPSYYENL	1424.5
-	1215	WYTSPSYWENL	1447.6
-	1	RYSLPPELSNM	1308.6
-	2	LYRSPSMPENL	1308.6

173	LYNSPSYFENL	1348.5	-
174	LYNSPSYYENL	1364.5	-
175	LYNSPSYWENL	1387.6	-
177	LYNSPSDPENL	1250.4	-
178	LYNSPSDFENL	1300.4	-
179	LYNSPSDYENL	1316.4	-
180	LYNSPSDWENL	1339.5	-
182	LYNSPSEPENL	1264.4	-
183	LYNSPSEFENL	1314.4	-
184	LYNSPSEYENL	1330.4	-
185	LYNSPSEWENL	1353.5	-
187	LYNSPSNPENL	1249.5	-
188	LYNSPSNFENL	1299.5	-
189	LYNSPSNYENL	1315.5	-
190	LYNSPSNWENL	1338.6	-
192	LYNSPSQPENL	1263.5	-
193	LYNSPSQFENL	1313.5	-
194	LYNSPSQYENL	1329.5	-
195	LYNSPSQWENL	1352.6	-
197	LYNSPSHPENL	1272.5	-
198	LYNSPSHFENL	1322.5	-
199	LYNSPSHYENL	1338.5	-
200	LYNSPSHWENL	1361.6	-
202	LYGSPSPMPENL	1209.5	-
203	LYGSPSMFENL	1259.5	-
204	LYGSPSMYENL	1275.5	-
205	LYGSPSMWENL	1298.6	-
207	LYGSPSFPENL	1225.4	-
208	LYGSPSFFENL	1275.4	-
209	LYGSPSFYENL	1291.4	-
210	LYGSPSFWENL	1314.5	-
212	LYGSPSYPENL	1241.4	-
213	LYGSPSYFENL	1291.4	-
214	LYGSPSYYENL	1307.4	-
215	LYGSPSYWENL	1330.5	-
217	LYGSPSDPENL	1193.3	-

218	LYGSPSDFENL	1243.3	-
219	LYGSPSDYENL	1259.3	-
220	LYGSPSDWENL	1282.4	-
222	LYGSPSEPENL	1207.3	-
223	LYGSPSEFENL	1257.3	-
224	LYGSPSEYENL	1273.3	-
225	LYGSPSEWENL	1296.4	-
227	LYGSPSNPENL	1192.4	-
228	LYGSPSNFENL	1242.4	-
229	LYGSPSNYENL	1258.4	-
230	LYGSPSNWENL	1281.5	-
232	LYGSPSQPENL	1206.4	-
233	LYGSPSQFENL	1256.4	-
234	LYGSPSQYENL	1272.4	-
235	LYGSPSQWENL	1295.5	-
237	LYGSPSHPENL	1215.4	-
238	LYGSPSHFENL	1265.4	-
239	LYGSPSHYENL	1281.4	-
240	LYGSPSHWENL	1304.5	-
242	LYASPSMPENL	1223.5	-
243	LYASPSMFENL	1273.5	-
244	LYASPSMYENL	1289.5	-
245	LYASPSMWENL	1312.6	-
247	LYASPSFPENL	1239.4	-
248	LYASPSFFENL	1289.4	-
249	LYASPSFYENL	1305.4	-
250	LYASPSFWENL	1328.5	-
252	LYASPSYPENL	1255.4	-
253	LYASPSYFENL	1305.4	-
254	LYASPSYYENL	1321.4	-
255	LYASPSYWENL	1344.5	-
257	LYASPSDPENL	1207.3	-
258	LYASPSDFENL	1257.3	-
259	LYASPSDYENL	1273.3	-
260	LYASPSDWENL	1296.4	-
262	LYASPSEPENL	1221.3	-

263	LYASPSEFENL	1271.3	-
264	LYASPSEYENL	1287.3	-
265	LYASPSEWENL	1310.4	-
267	LYASPSNPENL	1206.4	-
268	LYASPSNFENL	1256.4	-
269	LYASPSNYENL	1272.4	-
270	LYASPSNWENL	1295.5	-
272	LYASPSQPENL	1220.4	-
273	LYASPSQFENL	1270.4	-
274	LYASPSQYENL	1286.4	-
275	LYASPSQWENL	1309.5	-
277	LYASPSHPENL	1229.4	-
278	LYASPSHFENL	1279.4	-
279	LYASPSHYENL	1295.4	-
280	LYASPSHWENL	1318.5	-
282	LFRSPSMPENL	1292.6	-
283	LFRSPSMFENL	1342.6	-
284	LFRSPSMYENL	1358.6	-
285	LFRSPSMWENL	1381.7	-
287	LFRSPSFPENL	1308.5	-
288	LFRSPSFFENL	1358.5	-
289	LFRSPSFYENL	1374.5	-
290	LFRSPSFWENL	1397.6	-
292	LFRSPSYPENL	1324.5	-
293	LFRSPSYFENL	1374.5	-
294	LFRSPSYYENL	1390.5	-
295	LFRSPSYWENL	1413.6	-
297	LFRSPSDPENL	1276.4	-
298	LFRSPSDFENL	1326.4	-
299	LFRSPSDYENL	1342.4	-
300	LFRSPSDWENL	1365.5	-
302	LFRSPSEPENL	1290.4	-
303	LFRSPSEFENL	1340.4	-
304	LFRSPSEYENL	1356.4	-
305	LFRSPSEWENL	1379.5	-
307	LFRSPSNPENL	1275.5	-

308LFRSPSNFENL	1325.5	-
309LFRSPSNYENL	1341.5	-
310LFRSPSNWENL	1364.6	-
312LFRSPSQPENL	1289.5	-
313LFRSPSQFENL	1339.5	-
314LFRSPSQYENL	1355.5	-
315LFRSPSQWENL	1378.6	-
317LFRSPSHPENL	1298.5	-
318LFRSPSHFENL	1348.5	-
319LFRSPSHYENL	1364.5	-
320LFRSPSHWENL	1387.6	-
322LFSSPSMPENL	1224.3	-
323LFSSPSMFENL	1274.3	-
324LFSSPSMYENL	1290.3	-
325LFSSPSMWENL	1313.4	-
327LFSSPSFPENL	1240.2	-
328LFSSPSFFENL	1290.2	-
329LFSSPSFYENL	1306.2	-
330LFSSPSFWENL	1329.3	-
332LFSSPSYPENL	1256.2	-
333LFSSPSYFENL	1306.2	-
334LFSSPSYYENL	1322.2	-
335LFSSPSYWENL	1345.3	-
337LFSSPSDPENL	1208.1	-
338LFSSPSDFENL	1258.1	-
339LFSSPSDYENL	1274.1	-
340LFSSPSDWENL	1297.2	-
342LFSSPSEPENL	1222.1	-
343LFSSPSEFENL	1272.1	-
344LFSSPSEYENL	1288.1	-
345LFSSPSEWENL	1311.2	-
347LFSSPSNPENL	1207.2	-
348LFSSPSNFENL	1257.2	-
349LFSSPSNYENL	1273.2	-
350LFSSPSNWENL	1296.3	-
352LFSSPSQPENL	1221.2	-

353 LFSSPSQFENL	1271.2	-
354 LFSSPSQYENL	1287.2	-
355 LFSSPSQWENL	1310.3	-
357 LFSSPSHPENL	1230.2	-
358 LFSSPSHFENL	1280.2	-
359 LFSSPSHYENL	1296.2	-
360 LFSSPSHWENL	1319.3	-
362 LFTSPSMPENL	1237.5	-
363 LFTSPSMFENL	1287.5	-
364 LFTSPSMYENL	1303.5	-
365 LFTSPSMWENL	1326.6	-
367 LFTSPSFPENL	1253.4	-
368 LFTSPSFFENL	1303.4	-
369 LFTSPSFYENL	1319.4	-
370 LFTSPSFWENL	1342.5	-
372 LFTSPSYPENL	1269.4	+
373 LFTSPSYFENL	1319.4	+
374 LFTSPSYYENL	1335.4	+++
375 LFTSPSYWENL	1358.5	+
377 LFTSPSDPENL	1221.3	-
378 LFTSPSDFENL	1271.3	-
379 LFTSPSDYENL	1287.3	-
380 LFTSPSDWENL	1310.4	-
382 LFTSPSEPENL	1235.3	-
383 LFTSPSEFENL	1285.3	-
384 LFTSPSEYENL	1301.3	-
385 LFTSPSEWENL	1324.4	-
387 LFTSPSNPENL	1220.4	-
388 LFTSPSNFENL	1270.4	-
389 LFTSPSNYENL	1286.4	-
390 LFTSPSNWENL	1309.5	-
392 LFTSPSQPENL	1234.4	-
393 LFTSPSQFENL	1284.4	-
394 LFTSPSQYENL	1300.4	-
395 LFTSPSQWENL	1323.5	-
397 LFTSPSHPENL	1243.4	-

398 LFTSPSHFENL	1293.4	-
399 LFTSPSHYENL	1309.4	-
400 LFTSPSHWENL	1332.5	-
402 LFHSPSMPENL	1273.6	-
403 LFHSPSMFENL	1323.6	-
404 LFHSPSMYENL	1339.6	-
405 LFHSPSMWENL	1362.7	-
407 LFHSPSFPENL	1289.5	-
408 LFHSPSFFENL	1339.5	-
409 LFHSPSFYENL	1355.5	-
410 LFHSPSFWENL	1378.6	-
412 LFHSPSYPENL	1305.5	-
413 LFHSPSYFENL	1355.5	-
414 LFHSPSYYENL	1371.5	-
415 LFHSPSYWENL	1394.6	-
417 LFHSPSDPENL	1257.4	-
418 LFHSPSDFENL	1307.4	-
419 LFHSPSDYENL	1323.4	-
420 LFHSPSDWENL	1346.5	-
422 LFHSPSEPENL	1271.4	-
423 LFHSPSEFENL	1321.4	-
424 LFHSPSEYENL	1337.4	-
425 LFHSPSEWENL	1360.5	-
427 LFHSPSNPENL	1256.5	-
428 LFHSPSNFENL	1306.5	-
429 LFHSPSNYENL	1322.5	-
430 LFHSPSNWENL	1345.6	-
432 LFHSPSQPENL	1270.5	-
433 LFHSPSQFENL	1320.5	-
434 LFHSPSQYENL	1336.5	-
435 LFHSPSQWENL	1359.6	-
437 LFHSPSHPENL	1279.5	-
438 LFHSPSHFENL	1329.5	-
439 LFHSPSHYENL	1345.5	-
440 LFHSPSHWENL	1368.6	-
442 LFNPSMPENL	1250.6	-

443	LFNSPSMFENL	1300.6	-
444	LFNSPSMYENL	1316.6	-
445	LFNSPSMWENL	1339.7	-
447	LFNSPSFPENL	1266.5	-
448	LFNSPSFFENL	1316.5	-
449	LFNSPSFYENL	1332.5	-
450	LFNSPSFWENL	1355.6	-
452	LFNSPSYPENL	1282.5	-
453	LFNSPSYFENL	1332.5	-
454	LFNSPSYYENL	1348.5	-
455	LFNSPSYWENL	1371.6	-
457	LFNSPSDPENL	1234.4	-
458	LFNSPSDFENL	1284.4	-
459	LFNSPSDYENL	1300.4	-
460	LFNSPSDWENL	1323.5	-
462	LFNSPSEPENL	1248.4	-
463	LFNSPSEFENL	1298.4	-
464	LFNSPSEYENL	1314.4	-
465	LFNSPSEWENL	1337.5	-
467	LFNSPSNPENL	1233.5	-
468	LFNSPSNFENL	1283.5	-
469	LFNSPSNYENL	1299.5	-
470	LFNSPSNWENL	1322.6	-
472	LFNSPSQPENL	1247.5	-
473	LFNSPSQFENL	1297.5	-
474	LFNSPSQYENL	1313.5	-
475	LFNSPSQWENL	1336.6	-
477	LFNSPSHPENL	1256.5	-
478	LFNSPSHFENL	1306.5	-
479	LFNSPSHYENL	1322.5	-
480	LFNSPSHWENL	1345.6	-
482	LFGSPSMPENL	1193.5	-
483	LFGSPSMFENL	1243.5	-
484	LFGSPSMYENL	1259.5	-
485	LFGSPSMWENL	1282.6	-
487	LFGSPSFPENL	1209.4	-

488	LFGSPSFFENL	1259.4	-
489	LFGSPSFYENL	1275.4	-
490	LFGSPSFWENL	1298.5	-
492	LFGSPSYPENL	1225.4	-
493	LFGSPSYFENL	1275.4	-
494	LFGSPSYYENL	1291.4	-
495	LFGSPSYWENL	1314.5	-
497	LFGSPSDPENL	1177.3	-
498	LFGSPSDFENL	1227.3	-
499	LFGSPSDYENL	1243.3	-
500	LFGSPSDWENL	1266.4	-
502	LFGSPSEPENL	1191.3	-
503	LFGSPSEFENL	1241.3	-
504	LFGSPSEYENL	1257.3	-
505	LFGSPSEWENL	1280.4	-
507	LFGSPSNPENL	1176.4	-
508	LFGSPSNFENL	1226.4	-
509	LFGSPSNYENL	1242.4	-
510	LFGSPSNWENL	1265.5	-
512	LFGSPSQPENL	1190.4	-
513	LFGSPSQFENL	1240.4	-
514	LFGSPSQYENL	1256.4	-
515	LFGSPSQWENL	1279.5	-
517	LFGSPSHPENL	1199.4	-
518	LFGSPSHFENL	1249.4	-
519	LFGSPSHYENL	1265.4	-
520	LFGSPSHWENL	1288.5	-
522	LFASPSMPENL	1207.5	-
523	LFASPSMFENL	1257.5	-
524	LFASPSMYENL	1273.5	-
525	LFASPSMWENL	1296.6	-
527	LFASPSFPENL	1223.4	-
528	LFASPSFFENL	1273.4	-
529	LFASPSFYENL	1289.4	-
530	LFASPSFWENL	1312.5	-
532	LFASPSYPENL	1239.4	-

533 LFASPSYFENL	1289.4	-
534 LFASPSYYENL	1305.4	-
535 LFASPSYWENL	1328.5	-
537 LFASPSDPENL	1191.3	-
538 LFASPSDFENL	1241.3	-
539 LFASPSDYENL	1257.3	-
540 LFASPSDWENL	1280.4	-
542 LFASPSEPENL	1205.3	-
543 LFASPSEFENL	1255.3	-
544 LFASPSEYENL	1271.3	-
545 LFASPSEWENL	1294.4	-
547 LFASPSNPENL	1190.4	-
548 LFASPSNFENL	1240.4	-
549 LFASPSNYENL	1256.4	-
550 LFASPSNWENL	1279.5	-
552 LFASPSQPENL	1204.4	-
553 LFASPSQFENL	1254.4	-
554 LFASPSQYENL	1270.4	-
555 LFASPSQWENL	1293.5	-
557 LFASPSHPENL	1213.4	-
558 LFASPSHFENL	1263.4	-
559 LFASPSHYENL	1279.4	-
560 LFASPSHWENL	1302.5	-
562 FYRSPSMPENL	1342.6	-
563 FYRSPSMFENL	1392.6	-
564 FYRSPSMYENL	1408.6	-
565 FYRSPSMWENL	1431.7	-
567 FYRSPSFPENL	1358.5	-
568 FYRSPSFFENL	1408.5	-
569 FYRSPSFYENL	1424.5	-
570 FYRSPSFWENL	1447.6	-
572 FYRSPSYPENL	1374.5	-
573 FYRSPSYFENL	1424.5	-
574 FYRSPSYYENL	1440.5	-
575 FYRSPSYWENL	1463.6	-
577 FYRSPSDPENL	1326.4	-

578 FYRSPSDFENL	1376.4	-
579 FYRSPSDYENL	1392.4	-
580 FYRSPSDWENL	1415.5	-
582 FYRSPSEPENL	1340.4	-
583 FYRSPSEFENL	1390.4	-
584 FYRSPSEYENL	1406.4	-
585 FYRSPSEWENL	1429.5	-
587 FYRSPSNPENL	1325.5	-
588 FYRSPSNFENL	1375.5	-
589 FYRSPSNYENL	1391.5	-
590 FYRSPSNWENL	1414.6	-
592 FYRSPSQPENL	1339.5	-
593 FYRSPSQFENL	1389.5	-
594 FYRSPSQYENL	1405.5	-
595 FYRSPSQWENL	1428.6	-
597 FYRSPSHPENL	1348.5	-
598 FYRSPSHFENL	1398.5	-
599 FYRSPSHYENL	1414.5	-
600 FYRSPSHWENL	1437.6	-
602 FYSSPSMPENL	1274.3	-
603 FYSSPSMFENL	1324.3	-
604 FYSSPSMYENL	1340.3	-
605 FYSSPSMWENL	1363.4	-
607 FYSSPSFPENL	1290.2	-
608 FYSSPSFFENL	1340.2	-
609 FYSSPSFYENL	1356.2	-
610 FYSSPSFWENL	1379.3	-
612 FYSSPSYPENL	1306.2	-
613 FYSSPSYFENL	1356.2	-
614 FYSSPSYYENL	1372.2	-
615 FYSSPSYWENL	1395.3	-
617 FYSSPSDPENL	1258.1	-
618 FYSSPSDFENL	1308.1	-
619 FYSSPSDYENL	1324.1	-
620 FYSSPSDWENL	1347.2	-
622 FYSSPSEPENL	1272.1	-

623 FYSSPSEFENL	1322.1	-
624 FYSSPSEYENL	1338.1	-
625 FYSSPSEWENL	1361.2	-
627 FYSSPSNPENL	1257.2	-
628 FYSSPSNFENL	1307.2	-
629 FYSSPSNYENL	1323.2	-
630 FYSSPSNWENL	1346.3	-
632 FYSSPSQPENL	1271.2	-
633 FYSSPSQFENL	1321.2	-
634 FYSSPSQYENL	1337.2	-
635 FYSSPSQWENL	1360.3	-

637 FYSSPSHPENL	1280.2	+
638 FYSSPSHFENL	1330.2	+
639 FYSSPSHYENL	1346.2	+
640 FYSSPSHWENL	1369.3	+
642 FYTSPSMPENL	1287.5	+
643 FYTSPSMFENL	1337.5	+
644 FYTSPSMYENL	1353.5	+
645 FYTSPSMWENL	1376.6	+
647 FYTSPSFPENL	1303.4	+
648 FYTSPSFFENL	1353.4	+
649 FYTSPSFYENL	1369.4	+
650 FYTSPSFWENL	1392.5	+
652 FYTSPSYPENL	1319.4	+
653 FYTSPSYFENL	1369.4	+
654 FYTSPSYYENL	1385.4	+
655 FYTSPSYWENL	1408.5	+

657 FYTSPSDPENL	1271.3	-
658 FYTSPSDFENL	1321.3	-
659 FYTSPSDYENL	1337.3	-
660 FYTSPSDWENL	1360.4	-
662 FYTSPSEPENL	1285.3	-
663 FYTSPSEFENL	1335.3	-
664 FYTSPSEYENL	1351.3	-
665 FYTSPSEWENL	1374.4	-
667 FYTSPSNPENL	1270.4	-

668	FYTSPSNFENL	1320.4	-
669	FYTSPSNYENL	1336.4	-
670	FYTSPSNWENL	1359.5	-
672	FYTSPSQPENL	1284.4	-
673	FYTSPSQFENL	1334.4	-
674	FYTSPSQYENL	1350.4	-
675	FYTSPSQWENL	1373.5	-
677	FYTSPSHPENL	1293.4	-
678	FYTSPSHFENL	1343.4	-
679	FYTSPSHYENL	1359.4	-
680	FYTSPSHWENL	1382.5	-
682	FYHSPSMPENL	1323.6	-
683	FYHSPSMFENL	1373.6	-
684	FYHSPSMYENL	1389.6	-
685	FYHSPSMWENL	1412.7	-
687	FYHSPSFPENL	1339.5	-
688	FYHSPSFFENL	1389.5	-
689	FYHSPSFYENL	1405.5	-
690	FYHSPSFWENL	1428.6	-
692	FYHSPSYPENL	1355.5	-
693	FYHSPSYFENL	1405.5	-
694	FYHSPSYYENL	1421.5	-
695	FYHSPSYWENL	1444.6	-
697	FYHSPSDPENL	1307.4	-
698	FYHSPSDFENL	1357.4	-
699	FYHSPSDYENL	1373.4	-
700	FYHSPSDWENL	1396.5	-
702	FYHSPSEPENL	1321.4	-
703	FYHSPSEFENL	1371.4	-
704	FYHSPSEYENL	1387.4	-
705	FYHSPSEWENL	1410.5	-
707	FYHSPSNPENL	1306.5	-
708	FYHSPSNFENL	1356.5	-
709	FYHSPSNYENL	1372.5	-
710	FYHSPSNWENL	1395.6	-
712	FYHSPSQPENL	1320.5	-

713FYHSPSQFENL	1370.5	-
714FYHSPSQYENL	1386.5	-
715FYHSPSQWENL	1409.6	-
717FYHSPSHPENL	1329.5	-
718FYHSPSHFENL	1379.5	-
719FYHSPSHYENL	1395.5	-
720FYHSPSHWENL	1418.6	-
722FYNSPSMPENL	1300.6	-
723FYNSPSMFENL	1350.6	-
724FYNSPSMYENL	1366.6	-
725FYNSPSMWENL	1389.7	-
727FYNSPSFPENL	1316.5	-
728FYNSPSFFENL	1366.5	-
729FYNSPSFYENL	1382.5	-
730FYNSPSFWENL	1405.6	-
732FYNSPSYPENL	1332.5	-
733FYNSPSYFENL	1382.5	-
734FYNSPSYYENL	1398.5	-
735FYNSPSYWENL	1421.6	-
737FYNSPSDPENL	1284.4	-
738FYNSPSDFENL	1334.4	-
739FYNSPSDYENL	1350.4	-
740FYNSPSDWENL	1373.5	-
742FYNSPSEPENL	1298.4	-
743FYNSPSEFENL	1348.4	-
744FYNSPSEYENL	1364.4	-
745FYNSPSEWENL	1387.5	-
747FYNSPSNPENL	1283.5	-
748FYNSPSNFENL	1333.5	-
749FYNSPSNYENL	1349.5	-
750FYNSPSNWENL	1372.6	-
752FYNSPSQPENL	1297.5	-
753FYNSPSQFENL	1347.5	-
754FYNSPSQYENL	1363.5	-
755FYNSPSQWENL	1386.6	-
757FYNSPSHPENL	1306.5	-

758FYNSPSHFENL	1356.5	-
759FYNSPSHYENL	1372.5	-
760FYNSPSHWENL	1395.6	-
762FYGSPSMPENL	1243.5	-
763FYGSPSMFENL	1293.5	-
764FYGSPSMYENL	1309.5	-
765FYGSPSMWENL	1332.6	-
767FYGSPSFPENL	1259.4	-
768FYGSPSFFENL	1309.4	-
769FYGSPSFYENL	1325.4	-
770FYGSPSFWENL	1348.5	-
772FYGSPSYPENL	1275.4	-
773FYGSPSYFENL	1325.4	-
774FYGSPSYYENL	1341.4	-
775FYGSPSYWENL	1364.5	-
777FYGSPSDPENL	1227.3	-
778FYGSPSDFENL	1277.3	-
779FYGSPSDYENL	1293.3	-
780FYGSPSDWENL	1316.4	-
782FYGSPSEPENL	1241.3	-
783FYGSPSEFENL	1291.3	-
784FYGSPSEYENL	1307.3	-
785FYGSPSEWENL	1330.4	-
787FYGSPSNPENL	1226.4	-
788FYGSPSNFENL	1276.4	-
789FYGSPSNYENL	1292.4	-
790FYGSPSNWENL	1315.5	-
792FYGSPSQPENL	1240.4	-
793FYGSPSQFENL	1290.4	-
794FYGSPSQYENL	1306.4	-
795FYGSPSQWENL	1329.5	-
797FYGSPSHPENL	1249.4	-
798FYGSPSHFENL	1299.4	-
799FYGSPSHYENL	1315.4	-
800FYGSPSHWENL	1338.5	-
802FYASPSMPENL	1257.5	-

803 FYASPSMFENL	1307.5	-
804 FYASPSMYENL	1323.5	-
805 FYASPSMWENL	1346.6	-
807 FYASPSFPENL	1273.4	-
808 FYASPSFFENL	1323.4	-
809 FYASPSFYENL	1339.4	-
810 FYASPSFWENL	1362.5	-
812 FYASPSYPENL	1289.4	-
813 FYASPSYFENL	1339.4	-
814 FYASPSYYENL	1355.4	-
815 FYASPSYWENL	1378.5	-
817 FYASPSDPENL	1241.3	-
818 FYASPSDFENL	1291.3	-
819 FYASPSDYENL	1307.3	-
820 FYASPSDWENL	1330.4	-
822 FYASPSEPENL	1255.3	-
823 FYASPSEFENL	1305.3	-
824 FYASPSEYENL	1321.3	-
825 FYASPSEWENL	1344.4	-
827 FYASPSNPENL	1240.4	-
828 FYASPSNFENL	1290.4	-
829 FYASPSNYENL	1306.4	-
830 FYASPSNWENL	1329.5	-
832 FYASPSQPENL	1254.4	-
833 FYASPSQFENL	1304.4	-
834 FYASPSQYENL	1320.4	-
835 FYASPSQWENL	1343.5	-
837 FYASPSHPENL	1263.4	-
838 FYASPSHFENL	1313.4	-
839 FYASPSHYENL	1329.4	-
840 FYASPSHWENL	1352.5	-
842 FFRSPSMPENL	1326.6	-
843 FFRSPSMFENL	1376.6	-
844 FFRSPSMYENL	1392.6	-
845 FFRSPSMWENL	1415.7	-
847 FFRSPSFPENL	1342.5	-

848 FFRSPSFFENL	1392.5	-
849 FFRSPSFYENL	1408.5	-
850 FFRSPSFWENL	1431.6	-
852 FFRSPSYPENL	1358.5	-
853 FFRSPSYFENL	1408.5	-
854 FFRSPSYYENL	1424.5	-
855 FFRSPSYWENL	1447.6	-
857 FFRSPSDPENL	1310.4	-
858 FFRSPSDFENL	1360.4	-
859 FFRSPSDYENL	1376.4	-
860 FFRSPSDWENL	1399.5	-
862 FFRSPSEPENL	1324.4	-
863 FFRSPSEFENL	1374.4	-
864 FFRSPSEYENL	1390.4	-
865 FFRSPSEWENL	1413.5	-
867 FFRSPSNPENL	1309.5	-
868 FFRSPSNFENL	1359.5	-
869 FFRSPSNYENL	1375.5	-
870 FFRSPSNWENL	1398.6	-
872 FFRSPSQPENL	1323.5	-
873 FFRSPSQFENL	1373.5	-
874 FFRSPSQYENL	1389.5	-
875 FFRSPSQWENL	1412.6	-
877 FFRSPSHPENL	1332.5	-
878 FFRSPSHFENL	1382.5	-
879 FFRSPSHYENL	1398.5	-
880 FFRSPSHWENL	1421.6	-
882 FFSSPSMPENL	1258.3	-
883 FFSSPSMFENL	1308.3	-
884 FFSSPSMYENL	1324.3	-
885 FFSSPSMWENL	1347.4	-
887 FFSSPSFPENL	1274.2	-
888 FFSSPSFFENL	1324.2	-
889 FFSSPSFYENL	1340.2	-
890 FFSSPSFWENL	1363.3	-
892 FFSSPSYPENL	1290.2	-

893	FFSSPSYFENL	1340.2	-
894	FFSSPSYYENL	1356.2	-
895	FFSSPSYWENL	1379.3	-
897	FFSSPSDPENL	1242.1	-
898	FFSSPSDFENL	1292.1	-
899	FFSSPSDYENL	1308.1	-
900	FFSSPSDWENL	1331.2	-
902	FFSSPSEPENL	1256.1	-
903	FFSSPSEFENL	1306.1	-
904	FFSSPSEYENL	1322.1	-
905	FFSSPSEWENL	1345.2	-
907	FFSSPSNPENL	1241.2	-
908	FFSSPSNFENL	1291.2	-
909	FFSSPSNYENL	1307.2	-
910	FFSSPSNWENL	1330.3	-
912	FFSSPSQPENL	1255.2	-
913	FFSSPSQFENL	1305.2	-
914	FFSSPSQYENL	1321.2	-
915	FFSSPSQWENL	1344.3	-
917	FFSSPSHPENL	1264.2	-
918	FFSSPSHFENL	1314.2	-
919	FFSSPSHYENL	1330.2	-
920	FFSSPSHWENL	1353.3	-
922	FFTSPSMPENL	1271.5	-
923	FFTSPSMFENL	1321.5	-
924	FFTSPSMYENL	1337.5	-
925	FFTSPSMWENL	1360.6	-
927	FFTSPSFPENL	1287.4	-
928	FFTSPSFFENL	1337.4	-
929	FFTSPSFYENL	1353.4	-
930	FFTSPSFWENL	1376.5	-
932	FFTSPSYPENL	1303.4	-
933	FFTSPSYFENL	1353.4	-
934	FFTSPSYYENL	1369.4	-
935	FFTSPSYWENL	1392.5	-
937	FFTSPSDPENL	1255.3	-

938	FFTSPSDFENL	1305.3	-
939	FFTSPSDYENL	1321.3	-
940	FFTSPSDWENL	1344.4	-
942	FFTSPSEPENL	1269.3	-
943	FFTSPSEFENL	1319.3	-
944	FFTSPSEYENL	1335.3	-
945	FFTSPSEWENL	1358.4	-
947	FFTSPSNPENL	1254.4	-
948	FFTSPSNFENL	1304.4	-
949	FFTSPSNYENL	1320.4	-
950	FFTSPSNWENL	1343.5	-
952	FFTSPSQPENL	1268.4	-
953	FFTSPSQFENL	1318.4	-
954	FFTSPSQYENL	1334.4	-
955	FFTSPSQWENL	1357.5	-
957	FFTSPSHPENL	1277.4	-
958	FFTSPSHFENL	1327.4	-
959	FFTSPSHYENL	1343.4	-
960	FFTSPSHWENL	1366.5	-
962	FFHSPSMPENL	1307.6	-
963	FFHSPSMFENL	1357.6	-
964	FFHSPSMYENL	1373.6	-
965	FFHSPSMWENL	1396.7	-
967	FFHSPSFPENL	1323.5	-
968	FFHSPSFFENL	1373.5	-
969	FFHSPSFYENL	1389.5	-
970	FFHSPSFWENL	1412.6	-
972	FFHSPSYPENL	1339.5	-
973	FFHSPSYFENL	1389.5	-
974	FFHSPSYYENL	1405.5	-
975	FFHSPSYWENL	1428.6	-
977	FFHSPSDPENL	1291.4	-
978	FFHSPSDFENL	1341.4	-
979	FFHSPSDYENL	1357.4	-
980	FFHSPSDWENL	1380.5	-
982	FFHSPSEPENL	1305.4	-

983 FFHSPSEFENL	1355.4	-
984 FFHSPSEYENL	1371.4	-
985 FFHSPSEWENL	1394.5	-
987 FFHSPSNPENL	1290.5	-
988 FFHSPSNFENL	1340.5	-
989 FFHSPSNYENL	1356.5	-
990 FFHSPSNWENL	1379.6	-
992 FFHSPSQPENL	1304.5	-
993 FFHSPSQFENL	1354.5	-
994 FFHSPSQYENL	1370.5	-
995 FFHSPSQWENL	1393.6	-
997 FFHSPSHPENL	1313.5	-
998 FFHSPSHFENL	1363.5	-
999 FFHSPSHYENL	1379.5	-
1000 FFHSPSHWENL	1402.6	-
1002 FFNSPSMPENL	1284.6	-
1003 FFNSPSMFENL	1334.6	-
1004 FFNSPSMYENL	1350.6	-
1005 FFNSPSMWENL	1373.7	-
1007 FFNSPSFPENL	1300.5	-
1008 FFNSPSFFENL	1350.5	-
1009 FFNSPSFYENL	1366.5	-
1010 FFNSPSFWENL	1389.6	-
1012 FFNSPSYPENL	1316.5	-
1013 FFNSPSYFENL	1366.5	-
1014 FFNSPSYYENL	1382.5	-
1015 FFNSPSYWENL	1405.6	-
1017 FFNSPSDPENL	1268.4	-
1018 FFNSPSDFENL	1318.4	-
1019 FFNSPSDYENL	1334.4	-
1020 FFNSPSDWENL	1357.5	-
1022 FFNSPSEPENL	1282.4	-
1023 FFNSPSEFENL	1332.4	-
1024 FFNSPSEYENL	1348.4	-
1025 FFNSPSEWENL	1371.5	-
1027 FFNSPSNPENL	1267.5	-

1028	FFNSPSNFENL	1317.5	-
1029	FFNSPSNYENL	1333.5	-
1030	FFNSPSNWENL	1356.6	-
1032	FFNSPSQPENL	1281.5	-
1033	FFNSPSQFENL	1331.5	-
1034	FFNSPSQYENL	1347.5	-
1035	FFNSPSQWENL	1370.6	-
1037	FFNSPSHPENL	1290.5	-
1038	FFNSPSHFENL	1340.5	-
1039	FFNSPSHYENL	1356.5	-
1040	FFNSPSHWENL	1379.6	-
1042	FFGSPSPMPENL	1227.5	-
1043	FFGSPSPMFENL	1277.5	-
1044	FFGSPSPMYENL	1293.5	-
1045	FFGSPSPMWENL	1316.6	-
1047	FFGSPSPFPENL	1243.4	-
1048	FFGSPSPFFENL	1293.4	-
1049	FFGSPSPFYENL	1309.4	-
1050	FFGSPSPFWENL	1332.5	-
1052	FFGSPSPYPENL	1259.4	-
1053	FFGSPSPYFENL	1309.4	-
1054	FFGSPSPYYENL	1325.4	-
1055	FFGSPSPYWENL	1348.5	-
1057	FFGSPSPDPENL	1211.3	-
1058	FFGSPSPDFENL	1261.3	-
1059	FFGSPSPDYENL	1277.3	-
1060	FFGSPSPDWENL	1300.4	-
1062	FFGSPSPSEPENL	1225.3	-
1063	FFGSPSPSEFENL	1275.3	-
1064	FFGSPSPSEYENL	1291.3	-
1065	FFGSPSPSEWENL	1314.4	-
1067	FFGSPSPSNPENL	1210.4	-
1068	FFGSPSPSNFENL	1260.4	-
1069	FFGSPSPSNYENL	1276.4	-
1070	FFGSPSPSNWENL	1299.5	-
1072	FFGSPSPSQPENL	1224.4	-

1073 FFGSPSQFENL	1274.4	-
1074 FFGSPSQYENL	1290.4	-
1075 FFGSPSQWENL	1313.5	-
1077 FFGSPSHPENL	1233.4	-
1078 FFGSPSHFENL	1283.4	-
1079 FFGSPSHYENL	1299.4	-
1080 FFGSPSHWENL	1322.5	-
1082 FFASPSMPENL	1241.5	-
1083 FFASPSMFENL	1291.5	-
1084 FFASPSMYENL	1307.5	-
1085 FFASPSMWENL	1330.6	-
1087 FFASPSFPENL	1257.4	-
1088 FFASPSFFENL	1307.4	-
1089 FFASPSFYENL	1323.4	-
1090 FFASPSFWENL	1346.5	-
1092 FFASPSYPENL	1273.4	-
1093 FFASPSYFENL	1323.4	-
1094 FFASPSYYENL	1339.4	-
1095 FFASPSYWENL	1362.5	-
1097 FFASPSDPENL	1225.3	-
1098 FFASPSDFENL	1275.3	-
1099 FFASPSDYENL	1291.3	-
1100 FFASPSDWENL	1314.4	-
1102 FFASPSEPENL	1239.3	-
1103 FFASPSEFENL	1289.3	-
1104 FFASPSEYENL	1305.3	-
1105 FFASPSEWENL	1328.4	-
1107 FFASPSNPENL	1224.4	-
1108 FFASPSNFENL	1274.4	-
1109 FFASPSNYENL	1290.4	-
1110 FFASPSNWENL	1313.5	-
1112 FFASPSQPENL	1238.4	-
1113 FFASPSQFENL	1288.4	-
1114 FFASPSQYENL	1304.4	-
1115 FFASPSQWENL	1327.5	-
1117 FFASPSHPENL	1247.4	-

1118FFASPSHFENL 1297.4	-
1119FFASPSHYENL 1313.4	-
1120FFASPSHWENL 1336.5	-
1122WYRSPSMPENL 1381.7	+
1123WYRSPSMFENL 1431.7	+
1124WYRSPSMYENL 1447.7	++
1125WYRSPSMWENL 1470.8	++
1127WYRSPSPFENL 1397.6	++
1128WYRSPSPFENL 1447.6	++
1129WYRSPSFYENL 1463.6	+++
1130WYRSPSFWENL 1436.7	++
1132WYRSPSYPENL 1413.6	++
1133WYRSPSYFENL 1463.6	+
1134WYRSPSYENL 1479.6	++
1135WYRSPSYWENL 1502.7	+
1137WYRSPSDPENL 1365.5	-
1138WYRSPSDFENL 1415.5	-
1139WYRSPSDYENL 1431.5	-
1140WYRSPSDWENL 1454.6	-
1142WYRSPSEPENL 1379.5	-
1143WYRSPSEFENL 1429.5	-
1144WYRSPSEYENL 1445.5	-
1145WYRSPSEWENL 1468.6	-
1147WYRSPSNPENL 1364.6	-
1148WYRSPSNFENL 1414.6	-
1149WYRSPSNYENL 1430.6	-
1150WYRSPSNWENL 1453.7	-
1152WYRSPSQPENL 1378.6	-
1153WYRSPSQFENL 1428.6	-
1154WYRSPSQYENL 1444.6	-
1155WYRSPSQWENL 1467.7	-
1157WYRSPSHPENL 1387.6	-
1158WYRSPSHFENL 1437.6	-
1159WYRSPSHYENL 1453.6	-
1160WYRSPSHWENL 1476.7	-
1162WYSSPSMPENL 1313.4	-

1163 WYSSPSMFENL	1363.4	-
1164 WYSSPSMYENL	1379.4	-
1165 WYSSPSMWENL	1402.5	-
1167 WYSSPSFPENL	1329.3	-
1168 WYSSPSFFENL	1379.3	-
1169 WYSSPSFYENL	1395.3	-
1170 WYSSPSFWENL	1418.4	-
1172 WYSSPSYPENL	1345.3	-
1173 WYSSPSYFENL	1395.3	-
1174 WYSSPSYYENL	1411.3	-
1175 WYSSPSYWENL	1434.4	-
1177 WYSSPSDPENL	1297.2	-
1178 WYSSPSDFENL	1347.2	-
1179 WYSSPSDYENL	1363.2	-
1180 WYSSPSDWENL	1386.3	-
1182 WYSSPSEPENL	1311.2	-
1183 WYSSPSEFENL	1361.2	-
1184 WYSSPSEYENL	1377.2	-
1185 WYSSPSEWENL	1400.3	-
1187 WYSSPSNPENL	1296.3	-
1188 WYSSPSNFENL	1346.3	-
1189 WYSSPSNYENL	1362.3	-
1190 WYSSPSNWENL	1385.4	-
1192 WYSSPSQPENL	1310.3	-
1193 WYSSPSQFENL	1360.3	-
1194 WYSSPSQYENL	1376.3	-
1195 WYSSPSQWENL	1399.4	-
1197 WYSSPSHPENL	1319.3	-
1198 WYSSPSHFENL	1369.3	-
1199 WYSSPSHYENL	1385.3	-
1200 WYSSPSHWENL	1408.4	-
1202 WYTSPSPENL	1326.6	+
1203 WYTSPSMFENL	1376.6	+
1204 WYTSPSMYENL	1392.6	+
1205 WYTSPSMWENL	1415.7	+
1207 WYTSPSFPENL	1342.5	+

1208 WYTSPSFFENL	1392.5	+
1209 WYTSPSFYENL	1408.5	+
1210 WYTSPSFWENL	1431.6	+
1212 WYTSPSYPENL	1353.5	++
1213 WYTSPSYFENL	1408.5	+
1214 WYTSPSYYENL	1424.5	+
1215 WYTSPSYWENL	1447.6	+
1217 WYTSPSDPENL	1310.4	-
1218 WYTSPSDFENL	1360.4	-
1219 WYTSPSDYENL	1376.4	-
1220 WYTSPSDWENL	1399.5	-
1222 WYTSPSEPENL	1324.4	-
1223 WYTSPSEFENL	1374.4	-
1224 WYTSPSEYENL	1390.4	-
1225 WYTSPSEWENL	1413.5	-
1227 WYTSPSNPENL	1309.5	-
1228 WYTSPSNFENL	1359.5	-
1229 WYTSPSNYENL	1375.5	-
1230 WYTSPSNWENL	1398.6	-
1232 WYTSPSQPENL	1323.5	-
1233 WYTSPSQFENL	1373.5	-
1234 WYTSPSQYENL	1389.5	-
1235 WYTSPSQWENL	1412.6	-
1237 WYTSPSHPENL	1332.5	+
1238 WYTSPSHFENL	1382.5	+
1239 WYTSPSHYENL	1398.5	+
1240 WYTSPSHWENL	1421.6	+
1242 WYHSPSMPENL	1362.7	-
1243 WYHSPSMFENL	1412.7	-
1244 WYHSPSMYENL	1428.7	-
1245 WYHSPSMWENL	1451.8	-
1247 WYHSPSFPENL	1378.6	-
1248 WYHSPSFFENL	1428.6	-
1249 WYHSPSFYENL	1444.6	-
1250 WYHSPSFWENL	1467.7	-
1252 WYHSPSYPENL	1394.6	-

1253 WYHSPSYFENL	1444.6	-
1254 WYHSPSYYENL	1460.6	-
1255 WYHSPSYWENL	1483.7	-
1257 WYHSPSDPENL	1346.5	-
1258 WYHSPSDFENL	1396.5	-
1259 WYHSPSDYENL	1412.5	-
1260 WYHSPSDWENL	1435.6	-
1262 WYHSPSEPENL	1360.5	-
1263 WYHSPSEFENL	1410.5	-
1264 WYHSPSEYENL	1426.5	-
1265 WYHSPSEWENL	1449.6	-
1267 WYHSPSNPENL	1345.6	-
1268 WYHSPSNFENL	1395.6	-
1269 WYHSPSNYENL	1411.6	-
1270 WYHSPSNWENL	1434.7	-
1272 WYHSPSQPENL	1359.6	-
1273 WYHSPSQFENL	1409.6	-
1274 WYHSPSQYENL	1425.6	-
1275 WYHSPSQWENL	1448.7	-
1277 WYHSPSHPENL	1368.6	-
1278 WYHSPSHFENL	1418.6	-
1279 WYHSPSHYENL	1434.6	-
1280 WYHSPSHWENL	1457.7	-
1282 WYNPSMPENL	1339.7	-
1283 WYNPSMFENL	1389.7	-
1284 WYNPSMYENL	1405.7	-
1285 WYNPSMWENL	1428.8	-
1287 WYNPSFPENL	1355.6	-
1288 WYNPSFFENL	1405.6	-
1289 WYNPSFYENL	1421.6	-
1290 WYNPSFWENL	1444.7	-
1292 WYNPSYPENL	1371.6	-
1293 WYNPSYFENL	1421.6	-
1294 WYNPSYYENL	1437.6	-
1295 WYNPSYWENL	1460.7	-
1297 WYNPSDPENL	1323.5	-

1298 WYNPSDFENL	1373.5	-
1299 WYNPSDYENL	1389.5	-
1300 WYNPSDWENL	1412.6	-
1302 WYNPSSEPENL	1337.5	-
1303 WYNPSSEFENL	1387.5	-
1304 WYNPSSEYENL	1403.5	-
1305 WYNPSSEWENL	1426.6	-
1307 WYNPSNPENL	1322.6	-
1308 WYNPSNFENL	1372.6	-
1309 WYNPSNYENL	1388.6	-
1310 WYNPSNWENL	1411.7	-
1312 WYNPSQPENL	1336.6	-
1313 WYNPSQFENL	1386.6	-
1314 WYNPSQYENL	1402.6	-
1315 WYNPSQWENL	1425.7	-
1317 WYNPSHPENL	1345.6	-
1318 WYNPSHFENL	1395.6	-
1319 WYNPSHYENL	1411.6	-
1320 WYNPSHWENL	1434.7	-
1322 WYGSPSMPENL	1282.6	-
1323 WYGSPSMFENL	1332.6	-
1324 WYGSPSMYENL	1348.6	-
1325 WYGSPSMWENL	1371.7	-
1327 WYGSPSFPENL	1298.5	-
1328 WYGSPSFFENL	1348.5	-
1329 WYGSPSFYENL	1364.5	-
1330 WYGSPSFWENL	1387.6	-
1332 WYGSPSYPENL	1314.5	-
1333 WYGSPSYFENL	1364.5	-
1334 WYGSPSYYENL	1380.5	-
1335 WYGSPSYWENL	1403.6	-
1337 WYGSPSDPENL	1266.4	-
1338 WYGSPSDFENL	1316.4	-
1339 WYGSPSDYENL	1332.4	-
1340 WYGSPSDWENL	1355.5	-
1342 WYGSPSEPENL	1280.4	-

1343 WYGSPSEFENL	1330.4	-
1344 WYGSPSEYENL	1346.4	-
1345 WYGSPSEWENL	1369.5	-
1347 WYGSPSNPENL	1265.5	-
1348 WYGSPSNFENL	1315.5	-
1349 WYGSPSNYENL	1331.5	-
1350 WYGSPSNWENL	1354.6	-
1352 WYGSPSQPENL	1279.5	-
1353 WYGSPSQFENL	1329.5	-
1354 WYGSPSQYENL	1345.5	-
1355 WYGSPSQWENL	1368.6	-
1357 WYGSPSHPENL	1288.5	-
1358 WYGSPSHFENL	1338.5	-
1359 WYGSPSHYENL	1354.5	-
1360 WYGSPSHWENL	1377.6	-
1362 WYASPSMPENL	1296.6	-
1363 WYASPSMFENL	1346.6	-
1364 WYASPSMYENL	1362.6	-
1365 WYASPSMWENL	1385.7	-
1367 WYASPSFPENL	1312.5	-
1368 WYASPSFFENL	1362.5	-
1369 WYASPSFYENL	1378.5	-
1370 WYASPSFWENL	1401.6	-
1372 WYASPSYPENL	1328.5	-
1373 WYASPSYFENL	1378.5	-
1374 WYASPSYYENL	1394.5	-
1375 WYASPSYWENL	1417.6	-
1377 WYASPSDPENL	1280.4	-
1378 WYASPSDFENL	1330.4	-
1379 WYASPSDYENL	1346.4	-
1380 WYASPSDWENL	1369.5	-
1382 WYASPSEPENL	1294.4	-
1383 WYASPSEFENL	1344.4	-
1384 WYASPSEYENL	1360.4	-
1385 WYASPSEWENL	1383.5	-
1387 WYASPSNPENL	1279.5	-

1388 WYASPSNFENL	1329.5	-
1389 WYASPSNYENL	1345.5	-
1390 WYASPSNWENL	1368.6	-
1392 WYASPSQPENL	1293.5	-
1393 WYASPSQFENL	1343.5	-
1394 WYASPSQYENL	1359.5	-
1395 WYASPSQWENL	1382.6	-
1397 WYASPSHPENL	1302.5	-
1398 WYASPSHFENL	1352.5	-
1399 WYASPSHYENL	1368.5	-
1400 WYASPSHWENL	1391.6	-
1402 WFRSPSMPENL	1365.7	-
1403 WFRSPSMFENL	1415.7	-
1404 WFRSPSMYENL	1431.7	-
1405 WFRSPSMWENL	1454.8	-
1407 WFRSPSFPENL	1381.6	-
1408 WFRSPSFFENL	1431.6	-
1409 WFRSPSFYENL	1447.6	-
1410 WFRSPSFWENL	1470.7	-
1412 WFRSPSYPENL	1397.6	-
1413 WFRSPSYFENL	1447.6	-
1414 WFRSPSYYENL	1463.6	-
1415 WFRSPSYWENL	1486.7	-
1417 WFRSPSDPENL	1349.5	-
1418 WFRSPSDFENL	1399.5	-
1419 WFRSPSDYENL	1415.5	-
1420 WFRSPSDWENL	1438.6	-
1422 WFRSPSEPENL	1363.5	-
1423 WFRSPSEFENL	1413.5	-
1424 WFRSPSEYENL	1429.5	-
1425 WFRSPSEWENL	1452.6	-
1427 WFRSPSNPENL	1348.6	-
1428 WFRSPSNFENL	1398.6	-
1429 WFRSPSNYENL	1414.6	-
1430 WFRSPSNWENL	1437.7	-
1432 WFRSPSQPENL	1362.6	-

1433 WFRSPSQFENL	1412.6	-
1434 WFRSPSQYENL	1428.6	-
1435 WFRSPSQWENL	1451.7	-
1437 WFRSPSHPENL	1371.6	-
1438 WFRSPSHFENL	1421.6	-
1439 WFRSPSHYENL	1437.6	-
1440 WFRSPSHWENL	1460.7	-
1442 WFSSPSMPENL	1297.4	-
1443 WFSSPSMFENL	1347.4	-
1444 WFSSPSMYENL	1363.4	-
1445 WFSSPSMWENL	1386.5	-
1447 WFSSPSFPENL	1313.3	-
1448 WFSSPSFFENL	1363.3	-
1449 WFSSPSFYENL	1379.3	-
1450 WFSSPSFWENL	1402.4	-
1452 WFSSPSYPENL	1329.3	-
1453 WFSSPSYFENL	1379.3	-
1454 WFSSPSYYENL	1395.3	-
1455 WFSSPSYWENL	1418.4	-
1457 WFSSPSDPENL	1281.2	-
1458 WFSSPSDFENL	1331.2	-
1459 WFSSPSDYENL	1347.2	-
1460 WFSSPSDWENL	1370.3	-
1462 WFSSPSEPENL	1295.2	-
1463 WFSSPSEFENL	1345.2	-
1464 WFSSPSEYENL	1361.2	-
1465 WFSSPSEWENL	1384.3	-
1467 WFSSPSNPENL	1280.3	-
1468 WFSSPSNFENL	1330.3	-
1469 WFSSPSNYENL	1346.3	-
1470 WFSSPSNWENL	1369.4	-
1472 WFSSPSQPENL	1294.3	-
1473 WFSSPSQFENL	1344.3	-
1474 WFSSPSQYENL	1360.3	-
1475 WFSSPSQWENL	1383.4	-
1477 WFSSPSHPENL	1303.3	-

1478	WFSSPSHFENL	1353.3	-
1479	WFSSPSHYENL	1369.3	-
1480	WFSSPSHWENL	1392.4	-
1482	WFTSPSMPENL	1310.6	-
1483	WFTSPSMFENL	1360.6	-
1484	WFTSPSMYENL	1376.6	-
1485	WFTSPSMWENL	1399.7	-
1487	WFTSPSFPENL	1326.5	-
1488	WFTSPSFFENL	1376.5	-
1489	WFTSPSFYENL	1392.5	-
1490	WFTSPSFWENL	1415.6	-
1492	WFTSPSYPENL	1342.5	-
1493	WFTSPSYFENL	1392.5	-
1494	WFTSPSYYENL	1408.5	-
1495	WFTSPSYWENL	1431.6	-
1497	WFTSPSDPENL	1294.4	-
1498	WFTSPSDFENL	1344.4	-
1499	WFTSPSDYENL	1360.4	-
1500	WFTSPSDWENL	1383.5	-
1502	WFTSPSEPENL	1308.4	-
1503	WFTSPSEFENL	1358.4	-
1504	WFTSPSEYENL	1374.4	-
1505	WFTSPSEWENL	1397.5	-
1507	WFTSPSNPENL	1293.5	-
1508	WFTSPSNFENL	1343.5	-
1509	WFTSPSNYENL	1359.5	-
1510	WFTSPSNWENL	1382.6	-
1512	WFTSPSQPENL	1307.5	-
1513	WFTSPSQFENL	1357.5	-
1514	WFTSPSQYENL	1373.5	-
1515	WFTSPSQWENL	1396.6	-
1517	WFTSPSHPENL	1316.5	-
1518	WFTSPSHFENL	1366.5	-
1519	WFTSPSHYENL	1382.5	-
1520	WFTSPSHWENL	1405.6	-
1522	WFHSPSMPENL	1346.7	-

1523 WFHSPSMFENL	1396.7	-
1524 WFHSPSMYENL	1412.7	-
1525 WFHSPSMWENL	1435.8	-
1527 WFHSPSFPENL	1362.6	-
1528 WFHSPSFFENL	1412.6	-
1529 WFHSPSFYENL	1428.6	-
1530 WFHSPSFWENL	1451.7	-
1532 WFHSPSYPENL	1378.6	-
1533 WFHSPSYFENL	1428.6	-
1534 WFHSPSYYENL	1444.6	-
1535 WFHSPSYWENL	1467.7	-
1537 WFHSPSDPENL	1330.5	-
1538 WFHSPSDFENL	1380.5	-
1539 WFHSPSDYENL	1396.5	-
1540 WFHSPSDWENL	1419.6	-
1542 WFHSPSEPENL	1344.5	-
1543 WFHSPSEFENL	1394.5	-
1544 WFHSPSEYENL	1410.5	-
1545 WFHSPSEWENL	1433.6	-
1547 WFHSPSNPENL	1329.6	-
1548 WFHSPSNFENL	1379.6	-
1549 WFHSPSNYENL	1395.6	-
1550 WFHSPSNWENL	1418.7	-
1552 WFHSPSQPENL	1343.6	-
1553 WFHSPSQFENL	1393.6	-
1554 WFHSPSQYENL	1409.6	-
1555 WFHSPSQWENL	1432.7	-
1557 WFHSPSHPENL	1352.6	-
1558 WFHSPSHFENL	1402.6	-
1559 WFHSPSHYENL	1418.6	-
1560 WFHSPSHWENL	1441.7	-
1562 WFNSPSMPENL	1323.7	-
1563 WFNSPSMFENL	1373.7	-
1564 WFNSPSMYENL	1389.7	-
1565 WFNSPSMWENL	1412.8	-
1567 WFNSPSFPENL	1339.6	-

1568	WFNSP	SFFENL	1389.6	-
1569	WFNSP	SFYENL	1405.6	-
1570	WFNSP	SFWENL	1428.7	-
1572	WFNSP	SPYPENL	1355.6	-
1573	WFNSP	SYFENL	1405.6	-
1574	WFNSP	SYYENL	1421.6	-
1575	WFNSP	SYWENL	1444.7	-
1577	WFNSP	SDPENL	1307.5	-
1578	WFNSP	SDFENL	1357.5	-
1579	WFNSP	SDYENL	1373.5	-
1580	WFNSP	SDWENL	1396.6	-
1582	WFNSP	SEPENL	1321.5	-
1583	WFNSP	SEFENL	1371.5	-
1584	WFNSP	SEYENL	1387.5	-
1585	WFNSP	SEWENL	1410.6	-
1587	WFNSP	SNPENL	1306.6	-
1588	WFNSP	SNFENL	1356.6	-
1589	WFNSP	SNYENL	1372.6	-
1590	WFNSP	SNWENL	1395.7	-
1592	WFNSP	SQPENL	1320.6	-
1593	WFNSP	SQFENL	1370.6	-
1594	WFNSP	SQYENL	1386.6	-
1595	WFNSP	SQWENL	1409.7	-
1597	WFNSP	SHPENL	1329.6	-
1598	WFNSP	SHFENL	1379.6	-
1599	WFNSP	SHYENL	1395.6	-
1600	WFNSP	SHWENL	1418.7	-
1602	WFGSP	SPMPENL	1266.6	-
1603	WFGSP	SPMFENL	1316.6	-
1604	WFGSP	SPMYENL	1332.6	-
1605	WFGSP	SPMWENL	1355.7	-
1607	WFGSP	SFPENL	1282.5	-
1608	WFGSP	SFFENL	1332.5	-
1609	WFGSP	SFYENL	1348.5	-
1610	WFGSP	SFWENL	1371.6	-
1612	WFGSP	SPYPENL	1298.5	-

1613 WFGSPSYFENL	1348.5	-
1614 WFGSPSYYENL	1364.5	-
1615 WFGSPSYWENL	1387.6	-
1617 WFGSPSDPENL	1250.4	-
1618 WFGSPSDFENL	1300.4	-
1619 WFGSPSDYENL	1316.4	-
1620 WFGSPSDWENL	1339.5	-
1622 WFGSPSEPENL	1264.4	-
1623 WFGSPSEFENL	1314.4	-
1624 WFGSPSEYENL	1330.4	-
1625 WFGSPSEWENL	1353.5	-
1627 WFGSPSNPENL	1249.5	-
1628 WFGSPSNFENL	1299.5	-
1629 WFGSPSNYENL	1315.5	-
1630 WFGSPSNWENL	1338.6	-
1632 WFGSPSQPENL	1263.5	-
1633 WFGSPSQFENL	1313.5	-
1634 WFGSPSQYENL	1329.5	-
1635 WFGSPSQWENL	1352.6	-
1637 WFGSPSHPENL	1272.5	-
1638 WFGSPSHFENL	1322.5	-
1639 WFGSPSHYENL	1338.5	-
1640 WFGSPSHWENL	1361.6	-
1642 WFASPSMPENL	1280.6	-
1643 WFASPSMFENL	1330.6	-
1644 WFASPSMYENL	1346.6	-
1645 WFASPSMWENL	1369.7	-
1647 WFASPSFPENL	1296.5	-
1648 WFASPSFFENL	1346.5	-
1649 WFASPSFYENL	1362.5	-
1650 WFASPSFWENL	1385.6	-
1652 WFASPSYPENL	1312.5	-
1653 WFASPSYFENL	1362.5	-
1654 WFASPSYYENL	1378.5	-
1655 WFASPSYWENL	1401.6	-
1657 WFASPSDPENL	1264.4	-

1658	WFASPSDFENL	1314.4	-
1659	WFASPSDYENL	1330.4	-
1660	WFASPSDWENL	1353.5	-
1662	WFASPSEPENL	1278.4	-
1663	WFASPSEFENL	1328.4	-
1664	WFASPSEYENL	1344.4	-
1665	WFASPSEWENL	1367.5	-
1667	WFASPSNPENL	1263.5	-
1668	WFASPSNFENL	1313.5	-
1669	WFASPSNYENL	1329.5	-
1670	WFASPSNWENL	1352.6	-
1672	WFASPSQPENL	1277.5	-
1673	WFASPSQFENL	1327.5	-
1674	WFASPSQYENL	1343.5	-
1675	WFASPSQWENL	1366.6	-
1677	WFASPSHPENL	1286.5	-
1678	WFASPSHFENL	1336.5	-
1679	WFASPSHYENL	1352.5	-
1680	WFASPSHWENL	1375.6	-
1682	MYRSPSMPENL	1326.7	-
1683	MYRSPSMFENL	1376.7	-
1684	MYRSPSMYENL	1392.7	-
1685	MYRSPSMWENL	1415.8	-
1687	MYRSPSFPENL	1342.6	-
1688	MYRSPSFFENL	1392.6	-
1689	MYRSPSFYENL	1408.6	-
1690	MYRSPSFWENL	1431.7	-
1692	MYRSPSYPENL	1358.6	-
1693	MYRSPSYFENL	1408.6	-
1694	MYRSPSYYENL	1424.6	-
1695	MYRSPSYWENL	1447.7	-
1697	MYRSPSDPENL	1310.5	-
1698	MYRSPSDFENL	1360.5	-
1699	MYRSPSDYENL	1376.5	-
1700	MYRSPSDWENL	1399.6	-
1702	MYRSPSEPENL	1324.5	-

1703 MYRSPSEFENL	1374.5	-
1704 MYRSPSEYENL	1390.5	-
1705 MYRSPSEWENL	1413.6	-
1707 MYRSPSNPENL	1309.6	-
1708 MYRSPSNFENL	1359.6	-
1709 MYRSPSNYENL	1375.6	-
1710 MYRSPSNWENL	1398.7	-
1712 MYRSPSQPENL	1323.6	-
1713 MYRSPSQFENL	1373.6	-
1714 MYRSPSQYENL	1389.6	-
1715 MYRSPSQWENL	1412.7	-
1717 MYRSPSHPENL	1332.6	-
1718 MYRSPSHFENL	1382.6	-
1719 MYRSPSHYENL	1398.6	-
1720 MYRSPSHWENL	1421.7	-
1722 MYSSPSMPENL	1258.4	-
1723 MYSSPSMFENL	1308.4	-
1724 MYSSPSMYENL	1324.4	-
1725 MYSSPSMWENL	1347.5	-
1727 MYSSPSFPENL	1274.3	-
1728 MYSSPSFFENL	1324.3	-
1729 MYSSPSFYENL	1340.3	-
1730 MYSSPSFWENL	1363.4	-
1732 MYSSPSYPENL	1290.3	-
1733 MYSSPSYFENL	1340.3	-
1734 MYSSPSYYENL	1356.3	-
1735 MYSSPSYWENL	1379.4	-
1737 MYSSPSDPENL	1242.2	-
1738 MYSSPSDFENL	1292.2	-
1739 MYSSPSDYENL	1308.2	-
1740 MYSSPSDWENL	1331.3	-
1742 MYSSPSEPENL	1256.2	-
1743 MYSSPSEFENL	1306.2	-
1744 MYSSPSEYENL	1322.2	-
1745 MYSSPSEWENL	1345.3	-
1747 MYSSPSNPENL	1241.3	-

1748 MYSSPSNFENL	1291.3	-
1749 MYSSPSNYENL	1307.3	-
1750 MYSSPSNWENL	1330.4	-
1752 MYSSPSQPENL	1255.3	-
1753 MYSSPSQFENL	1305.3	-
1754 MYSSPSQYENL	1321.3	-
1755 MYSSPSQWENL	1344.4	-
1757 MYSSPSHPENL	1264.3	-
1758 MYSSPSHFENL	1314.3	-
1759 MYSSPSHYENL	1330.3	-
1760 MYSSPSHWENL	1353.4	-
1762 MYTSPSMPENL	1271.6	-
1763 MYTSPSMFENL	1321.6	-
1764 MYTSPSMYENL	1337.6	-
1765 MYTSPSMWENL	1360.7	-
1767 MYTSPSFPENL	1287.5	-
1768 MYTSPSFFENL	1337.5	-
1769 MYTSPSIFYENL	1353.5	-
1770 MYTSPSFWENL	1376.6	-
1772 MYTSPSYPENL	1303.5	-
1773 MYTSPSYFENL	1353.5	-
1774 MYTSPSYYENL	1369.5	-
1775 MYTSPSYWENL	1392.6	-
1777 MYTSPSDPENL	1255.4	-
1778 MYTSPSDFENL	1305.4	-
1779 MYTSPSDYENL	1321.4	-
1780 MYTSPSDWENL	1344.5	-
1782 MYTSPSEPENL	1269.4	-
1783 MYTSPSEFENL	1319.4	-
1784 MYTSPSEYENL	1335.4	-
1785 MYTSPSEWENL	1358.5	-
1787 MYTSPSNPENL	1254.5	-
1788 MYTSPSNFENL	1304.5	-
1789 MYTSPSNYENL	1320.5	-
1790 MYTSPSNWENL	1343.6	-
1792 MYTSPSQPENL	1268.5	-

1793	MYTSPSQFENL	1318.5	-
1794	MYTSPSQYENL	1334.5	-
1795	MYTSPSQWENL	1357.6	-
1797	MYTSPSHPENL	1277.5	-
1798	MYTSPSHFENL	1327.5	-
1799	MYTSPSHYENL	1343.5	-
1800	MYTSPSHWENL	1366.6	-
1802	MYHSPSMPENL	1307.7	-
1803	MYHSPSMFENL	1357.7	-
1804	MYHSPSMYENL	1373.7	-
1805	MYHSPSMWENL	1396.8	-
1807	MYHSPSFPENL	1323.6	-
1808	MYHSPSFFENL	1373.6	-
1809	MYHSPSFYENL	1389.6	-
1810	MYHSPSFWENL	1412.7	-
1812	MYHSPSYPENL	1339.6	-
1813	MYHSPSYFENL	1389.6	-
1814	MYHSPSYYENL	1405.6	-
1815	MYHSPSYWENL	1428.7	-
1817	MYHSPSDPENL	1291.5	-
1818	MYHSPSDFENL	1341.5	-
1819	MYHSPSDYENL	1357.5	-
1820	MYHSPSDWENL	1380.6	-
1822	MYHSPSEPENL	1305.5	-
1823	MYHSPSEFENL	1355.5	-
1824	MYHSPSEYENL	1371.5	-
1825	MYHSPSEWENL	1394.6	-
1827	MYHSPSNPENL	1290.6	-
1828	MYHSPSNFENL	1340.6	-
1829	MYHSPSNYENL	1356.6	-
1830	MYHSPSNWENL	1379.7	-
1832	MYHSPSQPENL	1304.6	-
1833	MYHSPSQFENL	1354.6	-
1834	MYHSPSQYENL	1370.6	-
1835	MYHSPSQWENL	1393.7	-
1837	MYHSPSHPENL	1313.6	-

1838	MYHSPSHFENL	1363.6	-
1839	MYHSPSHYENL	1379.6	-
1840	MYHSPSHWENL	1402.7	-
1842	MYNSPSPMPENL	1284.7	-
1843	MYNSPSPMFENL	1334.7	-
1844	MYNSPSPMYENL	1350.7	-
1845	MYNSPSPMWENL	1373.8	-
1847	MYNSPSPFPENL	1300.6	-
1848	MYNSPSPFFENL	1350.6	-
1849	MYNSPSPFYENL	1366.6	-
1850	MYNSPSPFWENL	1389.7	-
1852	MYNSPSPYPENL	1316.6	-
1853	MYNSPSPYFENL	1366.6	-
1854	MYNSPSPYYENL	1382.6	-
1855	MYNSPSPYWENL	1405.7	-
1857	MYNSPSPDPENL	1268.5	-
1858	MYNSPSPDFENL	1318.5	-
1859	MYNSPSPDYENL	1334.5	-
1860	MYNSPSPDWENL	1357.6	-
1862	MYNSPSEPENL	1282.5	-
1863	MYNSPSEFENL	1332.5	-
1864	MYNSPSEYENL	1348.5	-
1865	MYNSPSEWENL	1371.6	-
1867	MYNSPSPNPENL	1267.6	-
1868	MYNSPSPNFENL	1317.6	-
1869	MYNSPSPNYENL	1333.6	-
1870	MYNSPSPNWENL	1356.7	-
1872	MYNSPSPQPENL	1281.6	-
1873	MYNSPSPQFENL	1331.6	-
1874	MYNSPSPQYENL	1347.6	-
1875	MYNSPSPQWENL	1370.7	-
1877	MYNSPSPHPENL	1290.6	-
1878	MYNSPSPHFENL	1340.6	-
1879	MYNSPSPHYENL	1356.6	-
1880	MYNSPSPHWENL	1379.7	-
1882	MYGSPSPMPENL	1227.6	-

1883 MYGSPSMFENL	1277.6	-
1884 MYGSPSMYENL	1293.6	-
1885 MYGSPSMWENL	1316.7	-
1887 MYGSPSFPENL	1243.5	-
1888 MYGSPSFFENL	1293.5	-
1889 MYGSPSFYENL	1309.5	-
1890 MYGSPSFWENL	1332.6	-
1892 MYGSPSYPENL	1259.5	-
1893 MYGSPSYFENL	1309.5	-
1894 MYGSPSYYENL	1325.5	-
1895 MYGSPSYWENL	1348.6	-
1897 MYGSPSDPENL	1211.4	-
1898 MYGSPSDFENL	1261.4	-
1899 MYGSPSDYENL	1277.4	-
1900 MYGSPSDWENL	1300.5	-
1902 MYGSPSEPENL	1225.4	-
1903 MYGSPSEFENL	1275.4	-
1904 MYGSPSEYENL	1291.4	-
1905 MYGSPSEWENL	1314.5	-
1907 MYGSPSNPENL	1210.5	-
1908 MYGSPSNFENL	1260.5	-
1909 MYGSPSNYENL	1276.5	-
1910 MYGSPSNWENL	1299.6	-
1912 MYGSPSQPENL	1224.5	-
1913 MYGSPSQFENL	1274.5	-
1914 MYGSPSQYENL	1290.5	-
1915 MYGSPSQWENL	1313.6	-
1917 MYGSPSHPENL	1233.5	-
1918 MYGSPSHFENL	1283.5	-
1919 MYGSPSHYENL	1299.5	-
1920 MYGSPSHWENL	1322.6	-
1922 MYASPSMPENL	1241.6	-
1923 MYASPSMFENL	1291.6	-
1924 MYASPSMYENL	1307.6	-
1925 MYASPSMWENL	1330.7	-
1927 MYASPSFPENL	1257.5	-

1928 MYASPSFFENL	1307.5	-
1929 MYASPSFYENL	1323.5	-
1930 MYASPSFWENL	1346.6	-
1932 MYASPSYPENL	1273.5	-
1933 MYASPSYFENL	1323.5	-
1934 MYASPSYYENL	1339.5	-
1935 MYASPSYWENL	1362.6	-
1937 MYASPSDPENL	1225.4	-
1938 MYASPSDFENL	1275.4	-
1939 MYASPSDYENL	1291.4	-
1940 MYASPSDWENL	1314.5	-
1942 MYASPSEPENL	1239.4	-
1943 MYASPSEFENL	1289.4	-
1944 MYASPSEYENL	1305.4	-
1945 MYASPSEWENL	1328.5	-
1947 MYASPSNPENL	1224.5	-
1948 MYASPSNFENL	1274.5	-
1949 MYASPSNYENL	1290.5	-
1950 MYASPSNWENL	1313.6	-
1952 MYASPSQPENL	1238.5	-
1953 MYASPSQFENL	1288.5	-
1954 MYASPSQYENL	1304.5	-
1955 MYASPSQWENL	1327.6	-
1957 MYASPSHPENL	1247.5	-
1958 MYASPSHFENL	1297.5	-
1959 MYASPSHYENL	1313.5	-
1960 MYASPSHWENL	1336.6	-
1962 MFRSPSMPENL	1310.7	-
1963 MFRSPSMFENL	1360.7	-
1964 MFRSPSMYENL	1376.7	-
1965 MFRSPSMWENL	1399.8	-
1967 MFRSPSFPENL	1326.6	-
1968 MFRSPSFFENL	1376.6	-
1969 MFRSPSFYENL	1392.6	-
1970 MFRSPSFWENL	1415.7	-
1972 MFRSPSYPENL	1342.6	-

1973 MFRSPSYFENL	1392.6	-
1974 MFRSPSYYENL	1408.6	-
1975 MFRSPSYWENL	1431.7	-
1977 MFRSPSDPENL	1294.5	-
1978 MFRSPSDFENL	1344.5	-
1979 MFRSPSDYENL	1360.5	-
1980 MFRSPSDWENL	1383.6	-
1982 MFRSPSEPENL	1308.5	-
1983 MFRSPSEFENL	1358.5	-
1984 MFRSPSEYENL	1374.5	-
1985 MFRSPSEWENL	1397.6	-
1987 MFRSPSNPENL	1293.6	-
1988 MFRSPSNFENL	1343.6	-
1989 MFRSPSNYENL	1359.6	-
1990 MFRSPSNWENL	1382.7	-
1992 MFRSPSQPENL	1307.6	-
1993 MFRSPSQFENL	1357.6	-
1994 MFRSPSQYENL	1373.6	-
1995 MFRSPSQWENL	1396.7	-
1997 MFRSPSHPENL	1316.6	-
1998 MFRSPSHFENL	1366.6	-
1999 MFRSPSHYENL	1382.6	-
2000 MFRSPSHWENL	1405.7	-
2002 MFSSPSMPENL	1242.4	-
2003 MFSSPSMFENL	1292.4	-
2004 MFSSPSMYENL	1308.4	-
2005 MFSSPSMWENL	1331.5	-
2007 MFSSPSFPENL	1258.3	-
2008 MFSSPSFFENL	1308.3	-
2009 MFSSPSFYENL	1324.3	-
2010 MFSSPSFWENL	1347.4	-
2012 MFSSPSYPENL	1274.3	-
2013 MFSSPSYFENL	1324.3	-
2014 MFSSPSYYENL	1340.3	-
2015 MFSSPSYWENL	1363.4	-
2017 MFSSPSDPENL	1226.2	-

2018 MFSSPSDFENL	1276.2	-
2019 MFSSPSDYENL	1292.2	-
2020 MFSSPSDWENL	1315.3	-
2022 MFSSPSEPENL	1240.2	-
2023 MFSSPSEFENL	1290.2	-
2024 MFSSPSEYENL	1306.2	-
2025 MFSSPSEWENL	1329.3	-
2027 MFSSPSNPENL	1225.3	-
2028 MFSSPSNFENL	1275.3	-
2029 MFSSPSNYENL	1291.3	-
2030 MFSSPSNWENL	1314.4	-
2032 MFSSPSQPENL	1239.3	-
2033 MFSSPSQFENL	1289.3	-
2034 MFSSPSQYENL	1305.3	-
2035 MFSSPSQWENL	1328.4	-
2037 MFSSPSHPENL	1248.3	-
2038 MFSSPSHFENL	1298.3	-
2039 MFSSPSHYENL	1314.3	-
2040 MFSSPSHWENL	1337.4	-
2042 MFTSPSMPENL	1255.6	-
2043 MFTSPSMFENL	1305.6	-
2044 MFTSPSMYENL	1321.6	-
2045 MFTSPSMWENL	1344.7	-
2047 MFTSPSFPENL	1271.5	-
2048 MFTSPSFFENL	1321.5	-
2049 MFTSPSFYENL	1337.5	-
2050 MFTSPSFWENL	1360.6	-
2052 MFTSPSYPENL	1287.5	-
2053 MFTSPSYFENL	1337.5	-
2054 MFTSPSYYENL	1353.5	-
2055 MFTSPSYWENL	1376.6	-
2057 MFTSPSDPENL	1239.4	-
2058 MFTSPSDFENL	1289.4	-
2059 MFTSPSDYENL	1305.4	-
2060 MFTSPSDWENL	1328.5	-
2062 MFTSPSEPENL	1253.4	-

2063 MFTSPSEFENL	1303.4	-
2064 MFTSPSEYENL	1319.4	-
2065 MFTSPSEWENL	1342.5	-
2067 MFTSPSNPENL	1238.5	-
2068 MFTSPSNFENL	1288.5	-
2069 MFTSPSNYENL	1304.5	-
2070 MFTSPSNWENL	1327.6	-
2072 MFTSPSQPENL	1252.5	-
2073 MFTSPSQFENL	1302.5	-
2074 MFTSPSQYENL	1318.5	-
2075 MFTSPSQWENL	1341.6	-
2077 MFTSPSHPENL	1261.5	-
2078 MFTSPSHFENL	1311.5	-
2079 MFTSPSHYENL	1327.5	-
2080 MFTSPSHWENL	1350.6	-
2082 MFHSPSMPENL	1291.7	-
2083 MFHSPSMFENL	1341.7	-
2084 MFHSPSMYENL	1357.7	-
2085 MFHSPSMWENL	1380.8	-
2087 MFHSPSFPENL	1307.6	-
2088 MFHSPSFFENL	1357.6	-
2089 MFHSPSFYENL	1373.6	-
2090 MFHSPSFWENL	1396.7	-
2092 MFHSPSYPENL	1323.6	-
2093 MFHSPSYFENL	1373.6	-
2094 MFHSPSYYENL	1389.6	-
2095 MFHSPSYWENL	1412.7	-
2097 MFHSPSDPENL	1275.5	-
2098 MFHSPSDFENL	1325.5	-
2099 MFHSPSDYENL	1341.5	-
2100 MFHSPSDWENL	1364.6	-
2102 MFHSPSEPENL	1289.5	-
2103 MFHSPSEFENL	1339.5	-
2104 MFHSPSEYENL	1355.5	-
2105 MFHSPSEWENL	1378.6	-
2107 MFHSPSNPENL	1274.6	-

2108 MFHSPSNFENL	1324.6	-
2109 MFHSPSNYENL	1340.6	-
2110 MFHSPSNWENL	1363.7	-
2112 MFHSPSQPENL	1288.6	-
2113 MFHSPSQFENL	1338.6	-
2114 MFHSPSQYENL	1354.6	-
2115 MFHSPSQWENL	1377.7	-
2117 MFHSPSHPENL	1297.6	-
2118 MFHSPSHFENL	1347.6	-
2119 MFHSPSHYENL	1363.6	-
2120 MFHSPSHWENL	1386.7	-
2122 MFNSPSMPENL	1268.7	-
2123 MFNSPSMFENL	1318.7	-
2124 MFNSPSMYENL	1334.7	-
2125 MFNSPSMWENL	1357.8	-
2127 MFNSPSFPENL	1284.6	-
2128 MFNSPSFFENL	1334.6	-
2129 MFNSPSFYENL	1350.6	-
2130 MFNSPSFWENL	1373.7	-
2132 MFNSPSYPENL	1300.6	-
2133 MFNSPSYFENL	1350.6	-
2134 MFNSPSYYENL	1366.6	-
2135 MFNSPSYWENL	1389.7	-
2137 MFNSPSDPENL	1252.5	-
2138 MFNSPSDFENL	1302.5	-
2139 MFNSPSDYENL	1318.5	-
2140 MFNSPSDWENL	1341.6	-
2142 MFNSPSEPENL	1266.5	-
2143 MFNSPSEFENL	1316.5	-
2144 MFNSPSEYENL	1332.5	-
2145 MFNSPSEWENL	1355.6	-
2147 MFNSPSNPENL	1251.6	-
2148 MFNSPSNFENL	1301.6	-
2149 MFNSPSNYENL	1317.6	-
2150 MFNSPSNWENL	1340.7	-
2152 MFNSPSQPENL	1265.6	-

2153 MFNSPSQFENL	1315.6	-
2154 MFNSPSQYENL	1331.6	-
2155 MFNSPSQWENL	1354.7	-
2157 MFNSPSHPENL	1274.6	-
2158 MFNSPSHFENL	1324.6	-
2159 MFNSPSHYENL	1340.6	-
2160 MFNSPSHWENL	1363.7	-
2162 MFGSPSMPENL	1211.6	-
2163 MFGSPSMFENL	1261.6	-
2164 MFGSPSMYENL	1277.6	-
2165 MFGSPSMWENL	1300.7	-
2167 MFGSPSFPENL	1227.5	-
2168 MFGSPSFFENL	1277.5	-
2169 MFGSPSFYENL	1293.5	-
2170 MFGSPSFWENL	1316.6	-
2172 MFGSPSYPENL	1243.5	-
2173 MFGSPSYFENL	1293.5	-
2174 MFGSPSYYENL	1309.5	-
2175 MFGSPSYWENL	1332.6	-
2177 MFGSPSDPENL	1195.4	-
2178 MFGSPSDFENL	1245.4	-
2179 MFGSPSDYENL	1261.4	-
2180 MFGSPSDWENL	1284.5	-
2182 MFGSPSEPENL	1209.4	-
2183 MFGSPSEFENL	1259.4	-
2184 MFGSPSEYENL	1275.4	-
2185 MFGSPSEWENL	1298.5	-
2187 MFGSPSNPENL	1194.5	-
2188 MFGSPSNFENL	1244.5	-
2189 MFGSPSNYENL	1260.5	-
2190 MFGSPSNWENL	1283.6	-
2192 MFGSPSQPENL	1208.5	-
2193 MFGSPSQFENL	1258.5	-
2194 MFGSPSQYENL	1274.5	-
2195 MFGSPSQWENL	1297.6	-
2197 MFGSPSHPENL	1217.5	-

2198	MFGSPSHFENL	1267.5	-
2199	MFGSPSHYENL	1283.5	-
2200	MFGSPSHWENL	1306.6	-
2202	MFASPSMPENL	1225.6	-
2203	MFASPSMFENL	1275.6	-
2204	MFASPSMYENL	1291.6	-
2205	MFASPSMWENL	1314.7	-
2207	MFASPSFPENL	1241.5	-
2208	MFASPSFFENL	1291.5	-
2209	MFASPSFYENL	1307.5	-
2210	MFASPSFWENL	1330.6	-
2212	MFASPSYPENL	1257.5	-
2213	MFASPSYFENL	1307.5	-
2214	MFASPSYYENL	1323.5	-
2215	MFASPSYWENL	1346.6	-
2217	MFASPSDPENL	1209.4	-
2218	MFASPSDFENL	1259.4	-
2219	MFASPSDYENL	1275.4	-
2220	MFASPSDWENL	1298.5	-
2222	MFASPSEPENL	1223.4	-
2223	MFASPSEFENL	1273.4	-
2224	MFASPSEYENL	1289.4	-
2225	MFASPSEWENL	1312.5	-
2227	MFASPSNPENL	1208.5	-
2228	MFASPSNFENL	1258.5	-
2229	MFASPSNYENL	1274.5	-
2230	MFASPSNWENL	1297.6	-
2232	MFASPSQPENL	1222.5	-
2233	MFASPSQFENL	1272.5	-
2234	MFASPSQYENL	1288.5	-
2235	MFASPSQWENL	1311.6	-
2237	MFASPSHPENL	1231.5	-
2238	MFASPSHFENL	1281.5	-
2239	MFASPSHYENL	1297.5	-
2240	MFASPSHWENL	1320.6	-
2242	RYSLPPELSNM	1308.6	-

2243	A Y R S P S M P E N L	1266.5	-
2244	R Y R S P S M P E N L	1351.6	-
2245	N Y R S P S M P E N L	1309.6	-
2246	D Y R S P S M P E N L	1310.5	-
2247	C Y R S P S M P E N L	1298.6	-
2248	Q Y R S P S M P E N L	1323.6	-
2249	E Y R S P S M P E N L	1324.5	-
2250	G Y R S P S M P E N L	1252.5	-
2251	H Y R S P S M P E N L	1332.6	-
2252	I Y R S P S M P E N L	1308.6	-
2253	L Y R S P S M P E N L	1308.6	-
2254	K Y R S P S M P E N L	1323.6	-
2255	M Y R S P S M P E N L	1326.7	-
2256	F Y R S P S M P E N L	1342.6	-
2257	P Y R S P S M P E N L	1292.6	-
2258	S Y R S P S M P E N L	1283.3	-
2259	T Y R S P S M P E N L	1296.5	-
2260	W Y R S P S M P E N L	1381.7	-
2261	Y Y R S P S M P E N L	1358.6	-
2262	V Y R S P S M P E N L	1294.6	-
2263	L A R S P S M P E N L	1216.5	-
2264	L R R S P S M P E N L	1301.6	-
2265	L N R S P S M P E N L	1259.6	-
2266	L D R S P S M P E N L	1260.5	-
2267	L C R S P S M P E N L	1248.6	-
2268	L Q R S P S M P E N L	1273.6	-
2269	L E R S P S M P E N L	1274.5	-
2270	L G R S P S M P E N L	1202.5	-
2271	L H R S P S M P E N L	1282.6	-
2272	L I R S P S M P E N L	1258.6	-
2273	L L R S P S M P E N L	1258.6	-
2274	L K R S P S M P E N L	1273.6	+
2275	L M R S P S M P E N L	1276.7	-
2276	L F R S P S M P E N L	1292.6	-
2277	L P R S P S M P E N L	1242.6	-
2278	L S R S P S M P E N L	1233.3	-

2279	LTRSPSPENL	1246.5	-
2280	LWRSPSPENL	1331.7	-
2281	LYRSPSPENL	1308.6	-
2282	LVRSPSPENL	1244.6	-
2283	LYASPSMPENL	1223.5	-
2284	LYRSPSPENL	1308.6	-
2285	LYNSPSMPENL	1266.6	-
2286	LYDSPSPENL	1267.5	-
2287	LYCSPSPENL	1255.6	-
2288	LYQSPSPENL	1280.6	-
2289	LYESPSMPENL	1281.5	-
2290	LYGSPSPENL	1209.5	-
2291	LYHSPSPENL	1289.6	-
2292	LYISPSMPENL	1265.6	+
2293	LYLSPSPENL	1265.6	-
2294	LYKSPSPENL	1280.6	-
2295	LYMSPSPENL	1283.7	-
2296	LYFSPSPENL	1299.6	-
2297	LYPSPSPENL	1249.6	-
2298	LYSSPSMPENL	1240.3	-
2299	LYTSPSPENL	1253.5	-
2300	LYWSPSPENL	1338.7	-
2301	LYYSPSPENL	1315.6	-
2302	LYVSPSPENL	1251.6	-
2303	LYRSPSAPENL	1248.4	-
2304	LYRSPSRPENL	1333.5	-
2305	LYRSPSNPENL	1291.5	-
2306	LYRSPSDPENL	1292.4	-
2307	LYRSPSCPENL	1280.5	-
2308	LYRSPSQPENL	1305.5	-
2309	LYRSPSEPENL	1306.4	-
2310	LYRSPSGPENL	1234.4	-
2311	LYRSPSHPENL	1314.5	-
2312	LYRSPSIPENL	1290.5	-
2313	LYRSPSLPENL	1290.5	-
2314	LYRSPSKPENL	1305.5	-

2315	LYRSPSMPENL	1308.6	-
2316	LYRSPSFPENL	1324.5	-
2317	LYRSPSPPENL	1274.5	-
2318	LYRSPSSPENL	1265.2	-
2319	LYRSPSTPENL	1278.4	-
2320	LYRSPSWPENL	1363.6	-
2321	LYRSPSPYENL	1340.5	-
2322	LYRSPSPVENL	1276.5	-
2323	LYRSPSMAENL	1282.5	-
2324	LYRSPSMRENL	1367.6	-
2325	LYRSPSMNENL	1325.6	-
2326	LYRSPSMDENL	1326.5	-
2327	LYRSPSMCENL	1314.6	-
2328	LYRSPSMQENL	1339.6	-
2329	LYRSPSMEENL	1340.5	-
2330	LYRSPSMGENL	1268.5	-
2331	LYRSPSMHENL	1348.6	-
2332	LYRSPSMIENL	1324.6	-
2333	LYRSPSMLENL	1324.6	-
2334	LYRSPSMKENL	1339.6	-
2335	LYRSPSMMENL	1342.7	-
2336	LYRSPSMFENL	1358.6	-
2337	LYRSPSMPENL	1308.6	-
2338	LYRSPSMSENL	1299.3	-
2339	LYRSPSMTENL	1312.5	-
2340	LYRSPSMWENL	1397.7	-
2341	LYRSPSMYENL	1374.6	-
2342	LYRSPSMVENL	1310.6	+

Example 3: G2 abrogating peptides of the invention

The following example describes studies which identified exemplary G2 checkpoint-abrogating peptides of the invention. The following peptides of the invention were synthesized directly on membranes and tested in *in vitro* phosphorylation ("kination" assays, as described above.

Table 2

PEPTIDE	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	X ₉	X ₁₀	X ₁₁
AAA	L	A	R	S	A	S	M	P	E	A	L
RANDOMII	R	Y	S	L	P	P	E	L	S	N	M
S216A	L	Y	R	S	P	A	M	P	E	N	L
S216P	L	Y	R	S	P	S	M	P	E	N	L
YPN		Y	G	G	P	G	G	G	G	N	
YG7N		Y	G	G	G	G	G	G	G	N	
YG6N		Y	G	G	G	G	G	G		N	
YG5N		Y	G	G	G	G	G			N	
YPN		Y			P					N	
RPL			R					P			L
YGN		Y			G					N	

These peptides were tested in *in vitro* kination reactions. The oligopeptides were used as phosphorylation substrates; added kinases are involved in the cell cycle G2 checkpoint. Thus, a substance that inhibits the kination reaction can be a cell cycle G2 checkpoint abrogator. For the detection of the phosphorylation status of substrates in this screening method, isotope-labeled ATP and anti-phospho-peptides antibody can be used.

hChk1; hChk1 fusion proteins (MBP-peptide, GST-peptide), HuCds1/Chk2; HuCds1/Chk2 fusion proteins (MBP-peptide, GST-peptide); or, the cell extract from DNA damaged cells, can be used as the kinases in the screening assay.

The oligopeptides tested as substrates are Y X₂ X₃ P S X₆ X₇ X₈ N (X₂ through X₉, respectively; the first position (X₁) "Y" in this abbreviated nine residue motif corresponds to position X₂ in the eleven residue motif, described above) and variations thereof wherein amino acid residues at positions 2 (X₂) and position 3 (X₃) are Gly, Leu, Ser, or Arg; and the amino acid residue at position 6 through 8 are Gly, Leu, Ser, Met, Pro or Glu. Other tested oligopeptides sequence variations have amino acid residues at position 2 as Gly, Leu, Ser, or Arg; amino acid residues at position 3 as Gly, Leu or Ser; amino acid residues at position 6 as Gly, Met, Pro or Glu; amino acid residues at position 7 as Gly, Leu, or Pro; and, amino acid residues at position 8 as Gly, Met, Ser or Glu. In another variation the residue at

position 2 was Arg; position 3 was Ser; position 6 was Met; position 7 was Pro; and, position 8 was Glu.

The cells with the deficient cell cycle G1 checkpoint (such as a human leukemia-derived cell line Jurkat) were treated with a DNA damaging treatment. As the DNA damaging treatment, the cells were treated with bleomycin or other anti-cancer drugs. These drugs were added to the cell culture medium. Alternatively, the cells were irradiated with gamma irradiation. Peptides were added to these cells and the amount of DNA was determined some 10 to 48 hours after the DNA damage. The harvested cells were re-suspended with the solution that includes propidium iodide, RNase and NP-40 and analyzed by flow cytometer. If the oligopeptide "candidate substance" induces cells not to accumulate DNA at G2/M by this analysis, the result is positive and the substance potentially abrogated G2/M checkpoint.

Other screening methods can be used to identify selective inhibitors of the G2 cell cycle checkpoint. For, the cells are simultaneously treated with an oligopeptide "candidate phosphorylation substrate" and an M phase checkpoint activator, such as colchicine or nocodazol. The DNA content of the cells are analyzed some 10 to 48 hours after the treatment as described above. The candidates that do not disturb the accumulation of the cells at G2/M will be the selected G2 checkpoint abrogators in this screening method.

In one embodiment, G2 checkpoint abrogators at positions 2 and 3 the have amino acid residues Gly, Leu, Ser or Arg, and at position 5 to 8 are amino acid residues Ser, Gly, Met, Pro or Glu.

In one embodiment of the invention the compositions are enhancers or augmenters of a DNA damaging anti-cancer treatment. By treating cancer cells simultaneously or sequentially with an anti-cancer treatment and a G2 checkpoint inhibiting composition of the invention, one can effectively kill the cancer cells. Since the most human cancer cells do not have an intact G1 checkpoint, the abrogation of the G2 checkpoint by a G2 checkpoint inhibiting composition of the invention will effectively kill the cancer cells that are treated with a DNA damaging method. The compositions of the invention can be directly used as a drug (e.g., a pharmaceutical compositions) or these oligopeptides could be expressed recombinantly *in vivo*, e.g., from a virus vector or other expression vector, e.g., a plasmid, as an *in vivo* gene therapy.

Jurkat cells were cultured in 10% fetal calf serum with a medium (RPMI 1640) at 37°C/5% CO₂ with: bleomycin at 20 µg/ml; bleomycin at 20 µg/ml and the peptide “4aa” (amino acid sequence is GGSPSM); bleomycin at 20 µg/ml and the peptide AAA (Table 1); bleomycin at 20 µg/ml and the peptide YNP (Table 1). The amount of DNA was analyzed at 0, 6, 12, 24 hours after the addition of ten microgram of bleomycin with or without the oligopeptides “4aa,” “YNP” and “AAA.” The DNA quantity was analyzed by a flow cytometer (FACS) after the addition of a solution comprising propidium iodide, RNase and NP-40.

The results are shown in Figure 6. The left panels are actual results of flow cytometer (FACS) analysis. The right panel indicates the population of cells in each of the cell cycle phases (sub G1, G1, S, and G2/M). The results indicated that YNP peptide abrogated the G2 checkpoint because the cells do not accumulate at G2/M phases.

In another experiment, an M phase checkpoint activator, colchicine, was used instead of bleomycin: colchicine at 2.5 µg/ml; colchicine at 2.5 µg/ml and the peptide “4aa”; colchicine at 2.5 µg/ml and the peptide AAA (Table 1); colchicine at 2.5 µg/ml and the peptide YNP (Table 1), and no treatment. The results are shown in Figure 7. None of the above tested oligopeptides (Table 1), including, YPN, affected the accumulation of the colchicine-treated cells at the G2/M phase. These data indicated that YPN specifically abrogated the cell cycle at the G2 checkpoint.

Peptides which were tested and the results of these experiments are further summarized in Figures 8 and 9.

Example 4: Peptides of the invention sensitize cancer cells in *in vivo* animal model

The following example describes studies in an art-accepted animal model which demonstrated that exemplary peptides of the invention are effective agents for selectively sensitizing cancer cells to DNA damaging agents. In particular, nude mouse studies demonstrated the *in vivo* efficacy of the compositions and methods of the invention.

Human colon cancer cell line SW620 were injected subcutaneously into 3 week old Balb/c nude mouse (1x10⁸ cells per mouse). Some two weeks after the injection, the established subcutaneous tumors of diameter 2 to 4 mm were resected and transplanted to syngeneic mice. One week after the transplantation, the injection of cisplatin (CDDP) and peptides (TAT-control and TAT-S216, see Table 1) was started. The peptides were in the

form of recombinant fusion proteins, with TAT being the protein transduction domain having the sequence YGRKKRRQRRR.

Cisplatin (CDDP) at 6 mg/kg was injected once a week into peritoneum. Peptides (at 100 nM) were injected into tumor twice a week. Relative tumor weights were
5 assessed at 3 and 5 weeks. The results are shown in Figure 10, upper panel. Similar experiments were performed with 5-FU instead of cisplatin. The results are shown in Figure 8, lower panel. As shown in Figure 10, the S216-containing fusion protein effectively sensitized the cancer cells to a DNA damaging agent administered to the animal *in vivo*.

Similar experiments were performed with cisplatin (CDDP) and another
10 exemplary peptide of the invention, "random II" or "R-II" (see Table 1). As with S216, RII peptide was in the form of a recombinant fusion protein with TAT. The relative volume of the transplanted subcutaneous tumor with or without cisplatin ("CDDP"), CDDP plus DMSO, CDDP plus TAT-FLAG or CDDP plus TAT-Random II peptide was determined. As shown in Figure 11, the R-II containing fusion protein effectively sensitized the cancer cells
15 to a DNA damaging agent administered to the animal *in vivo*.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope
20 of the following claims.